

ROMANIAN JOURNAL of DIABETES, NUTRITION AND METABOLIC DISEASES

Original Research

CHANGES IN THE TRANSCRIPTIONAL ACTIVITY OF THE LYMPHOCYTE HOMING REGULATORY GENES MADCAM1, CXCR3, CCR7 AND S1PR1 AFFECT STRUCTURE OF THE POPULATION OF T-BET⁺, ROR γ T⁺ AND FOXP3⁺ CELLS IN MESENTERIC LYMPH NODES IN OFF-SPRING OF RATS WITH EXPERIMENTAL GESTATIONAL DIABETES

Tatjana Prozorova, Oleksandr Tokarsky, Larisa Fedoniuk, Viktoriia Harbuzova, Oksana Morozova, Alexander Egorov, Alexandr Kamyshnyi

SIRTUIN 1, BUT NOT OSTEOCALCIN, CORRELATES WITH LIPID ACCUMULATION PRODUCT, VISCERAL ADIPOSITY AND ATHEROGENICITY INDICES IN NEWLY DIAGNOSED PREDIABETES-METABOLIC SYNDROME PATIENTS

Violet Kasabri, Abba AlBsoul-Younes, Omar Rizik, Maysa Suyagh, Kashma Omed, Govand Yassin, Sundus AlAlawi

THE ROLE OF VITAMIN D IN TREATMENT OF POLYCYSTIC OVARY SYNDROME DEPENDING ON PHENOTYPE

Halyna Suslyk, Alina Urbanovych, Orysia Lishchuk, Krystyna Kozlovska

ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS OF USING ALPHA-TOCOPHEROL IN CELL CULTURE OF THE PAROTID GLAND UNDER CONDITIONS SIMILAR TO DIABETES MELLITUS

Victor Augusto Ramos Fernandes, Raphael Oliveira Ramos Franco Netto, Felipe Lovaglio Belozo, Eduardo José Caldeira

Case Report

DIGITAL AND CONVENTIONAL APPROACH FOR THE TREATMENT OF SEVERE ANTERIOR DENTAL WEAR IN A TYPE 2 DIABETES PATIENT

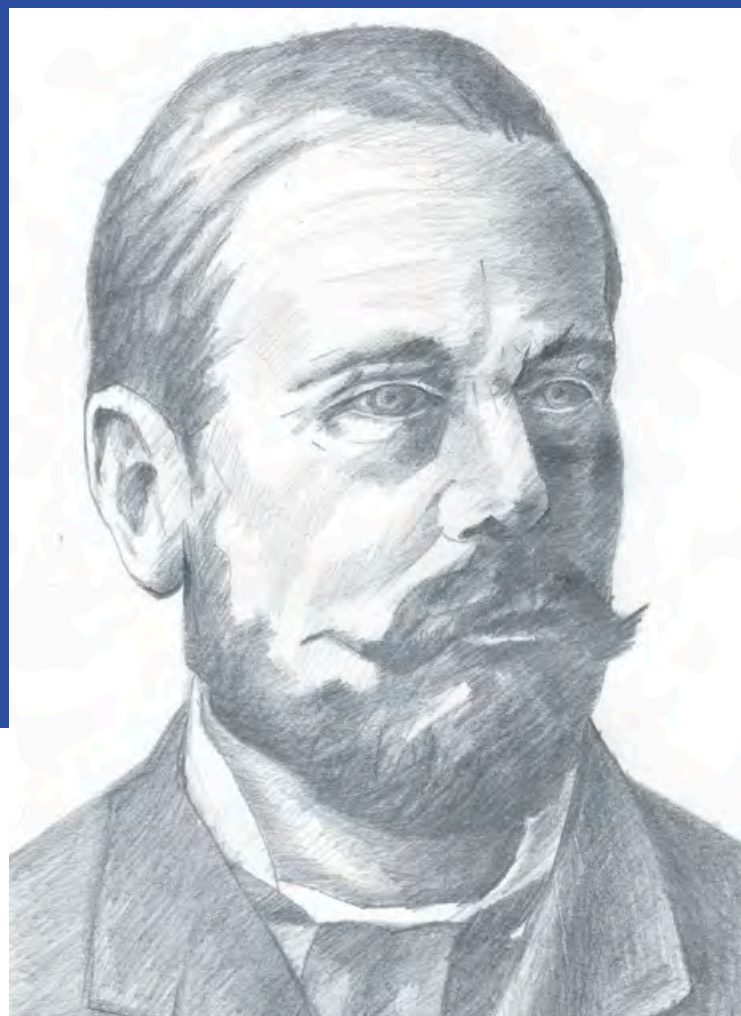
Raul Rotar, Codruta Ilie, Anca Jivanescu

Review

THE HANDHELD INFRARED THERMOMETRY IN THE DIABETIC FOOT – USEFUL BUT DEBATABLE TECHNIQUE

Elena-Rodica Perciun, Alexandru-Madalin Buse

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Editor-in-Chief: Cristian GUJA, MD, PhD

Carol Davila University of Medicine and Pharmacy, Bucharest, Romania
'N.C. Paulescu' National Institute of Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania,
5-7 Ion Movila Street, Bucharest 020475, Romania. Tel. +40 21 210 6460, Fax: +40 21 210 2295
E-mail: cristian.guja@b.astral.ro

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E-mail: bogdan.timar@rjdnmd.org

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Dragos Cretoiu, Bucharest, Romania
E-mail: journal@sanatateapress.ro

Assistant Editor

Roxana Stoica, Timisoara, Romania
E-mail: roxana.stoica@rjdnmd.org

Assistant Editor

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E-mail: sandra.lazar@rjdnmd.org

Assistant Editor

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E-mail: teodor.salmen@rjdnmd.org

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9C Alexandru Moruzzi street, Bucharest 031152, Romania. Tel.: +40 31 710 88 01, Fax: +40 031 710 88 02
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Jardiance® (empagliflozin) comprimate filmate 10 mg, 25 mg. Indicații terapeutice: Tratamentul adulților cu diabet zaharat tip 2 insuficient controlat, ca adjuvant la dietă și exercițiul fizic: ca monoterapie atunci când metformina este considerată inadecvată din cauza intoleranței; în plus față de alte medicamente pentru tratamentul diabetului zaharat. **Doze și mod de administrare:** Doza inițială este de 10 mg o dată pe zi (în monoterapie și în terapie asociată suplimentară cu alte medicamente pentru tratamentul diabetului zaharat). La pacienții care tolerează empagliflozin 10 mg o dată pe zi, care prezintă RFG ≥ 60 ml/min/1,73 m² și care necesită un control glicemic mai strict, doza poate fi crescută la 25 mg o dată pe zi. Doza zilnică maximă este de 25 mg. Când empagliflozin este utilizat în asociere cu o sulfoniluree sau cu insulină, se poate avea în vedere o doză mai mică de sulfoniluree sau de insulină, pentru a reduce riscul de hipoglicemie. **IR** Nu este necesară ajustarea dozei la pacienți cu RFG ≥ 60 ml/min/1,73 m² sau ClCr ≥ 60 ml/min. Administrarea nu trebuie inițiată la pacienți cu RFG < 60 ml/min/1,73 m² sau ClCr < 60 ml/min. La pacienții la care valorile RFG scad în mod persistent sub 60 ml/min/1,73 m² sau ClCr sub 60 ml/min, doza trebuie ajustată sau menținută la 10 mg o dată pe zi. Administrarea trebuie întreruptă la pacienții cu valori ale RFG aflate persistent sub 45 ml/min/1,73 m² sau cu valori ale ClCr aflate persistent sub 45 ml/min. Nu trebuie utilizat la pacienții cu BRST sau cărora li se efectuează dializă. **IH** Nu este necesară ajustarea dozei la pacienți cu IH. Nu se recomandă utilizarea la pacienți cu IH severă. **Vârșnici** La pacienții cu vârsta de 75 ani și peste, trebuie avut în vedere un risc crescut de depleție volemică. Nu se recomandă începerea tratamentului cu empagliflozin la pacienții cu vârsta de 85 ani și peste. **Copii și adolescenți** La acest grup de pacienți, siguranța și eficacitatea administrării empagliflozin nu au fost încă stabilite. Nu sunt disponibile date. **Mod de administrare** Comprimatele pot fi administrate cu sau fără alimente, înghițite întregi cu apă. Dacă se omite o doză, aceasta trebuie administrată imediat ce pacientul își aduce aminte. În aceeași zi nu trebuie administrată o doză dublă. **Contraindicații:** Hipersensibilitate la substanța activă sau la oricare dintre excipienți. **Atenționări și precauții speciale pentru utilizare:** Au fost raportate cazuri rare de CAD, inclusiv cazuri cu risc vital și cazuri letale la pacienți tratați cu inhibitori de SGLT2, inclusiv empagliflozin. Într-o serie de cazuri, natura afecțiunii a fost atipică, cu valori ale glicemiei mai mici de 14 mmol/l (250 mg/dl). Nu se cunoaște dacă CAD are o probabilitate mai mare de apariție în cazul utilizării dozelor mai mari de empagliflozin. Riscul de CAD trebuie luat în considerare în cazul apariției unor simptome nespecifice. Pacienții trebuie evaluați pentru depistarea CAD imediat ce apar aceste simptome, indiferent de valorile glicemiei. La pacienții unde se suspectează sau este diagnosticată CAD, tratamentul trebuie întrerupt imediat. Tratamentul trebuie întrerupt la pacienții care au fost spitalizați pentru intervenții chirurgicale majore sau afecțiuni medicale acute grave. La acești pacienți se recomandă monitorizarea cetonelor. Se preferă măsurarea valorii cetonelor din sânge, față de valoarea din urină. Tratamentul cu empagliflozin poate fi reînceput când valorile cetonelor s-au normalizat și starea pacientului a fost stabilizată. Înainte de a iniția tratamentul, trebuie luate în considerare antecedentele pacientului care pot predispuce la CAD. Nu se recomandă reluarea tratamentului cu inhibitor de SGLT2 la pacienții cu antecedente de CAD dezvoltată în timpul tratamentului cu inhibitor de SGLT2 decât dacă a fost identificat și rezolvat un alt factor precipitant evident. Siguranța și eficacitatea empagliflozin la pacienții cu diabet de tip 1 nu a fost stabilită și empagliflozin nu trebuie utilizat pentru tratarea pacienților cu diabet de tip 1. **Monitorizarea funcției renale:** Înainte de începerea tratamentului și periodic în timpul tratamentului, cel puțin anual; înainte de începerea tratamentului concomitent cu orice medicament care poate avea impact negativ asupra funcției renale. **Leziuni hepatice** Cazuri de leziuni hepatice au fost raportate atunci când s-a administrat empagliflozin în cadrul studiilor clinice. Nu s-a stabilit o relație de cauzalitate între empagliflozin și leziunea hepatică. **Risc de depleție volemică** Se impune prudență la pacienții la care scăderea tensiunii arteriale indusă de empagliflozin ar putea prezenta un risc. Până la corectarea pierderii de lichide, se va avea în vedere întreruperea temporară a tratamentului cu empagliflozin. **Infecții ale căilor urinare** În cadrul unor studii cumulate controlate cu placebo, frecvența infecțiilor căilor urinare raportate a fost similară la pacienții cărora li s-a administrat tratament cu empagliflozin 25 mg și placebo și mai mare la pacienții cărora li s-a administrat tratament cu empagliflozin 10 mg. La pacienții cărora li s-a administrat tratament cu empagliflozin s-au raportat ulterior puneri pe piață cazuri de infecții complicate ale căilor urinare. Întreruperea temporară a tratamentului cu empagliflozin trebuie avută în vedere la pacienții cu infecții complicate ale căilor urinare. **Fasceită necrozantă care afectează perineul (gangrena Fournier)** După punerea pe piață, s-au raportat cazuri de fasceită necrozantă care afectează perineul (afecțiune cunoscută și ca gangrena Fournier) la pacienți de ambele sexe tratați cu inhibitori de SGLT2. Este un eveniment rar, însă grav, care poate pune în pericol viața și care necesită intervenție chirurgicală urgentă și tratament cu antibiotice. În cazul în care există suspiciunea de gangrenă Fournier, se va întrerupe administrarea Jardiance® și se va institui imediat tratament (inclusiv antibiotice și debridare chirurgicală). **IC** Experiența la pacienți cu clasa NYHA I-II este limitată; nu există experiență în studiile clinice cu empagliflozin la pacienți cu clasa NYHA III-IV. În cadrul studiului EMPA-REG OUTCOME, la 10,1% dintre pacienți s-a raportat insuficiență cardiacă la momentul inițial. Scăderea numărului de decese de etiologie cardiovasculară la acești pacienți a fost similară cu cea înregistrată la populația de studiu totală. **Evaluarea analizelor de laborator ale urinei** Pacienții care urmează tratament cu Jardiance® vor avea glicozurie pozitivă. **Lactoză** Comprimatul conține lactoză. Pacienții cu afecțiuni ereditare rare de intoleranță la galactoză, deficit de lactază Lapp sau sindrom de malabsorbție a glucozei-galactozei nu trebuie să utilizeze acest medicament. **Fertilitatea, sarcina și alăptarea:** Datele provenite din utilizarea empagliflozinului la gravide sunt inexistente. Este de preferat să se evite utilizarea Jardiance® în timpul sarcinii. Nu trebuie utilizat în timpul alăptării. Nu s-au efectuat studii privind efectele Jardiance® asupra fertilității la om. Studiile la animale nu au evidențiat efecte dăunătoare directe sau indirecte cu privire la fertilitate. **Efecte asupra capacității de a conduce vehicule și de a folosi utilaje:** Jardiance® are influență mică asupra capacității de a conduce vehicule și de a folosi utilaje. Pacienții trebuie sfătuiți să ia măsuri de precauție pentru a evita hipoglicemia atunci când conduc vehicule și folosesc utilaje, în special atunci când Jardiance® este utilizat în asociere cu o sulfoniluree și/sau insulină. **Reacții adverse (RA):** Siguranța administrării empagliflozinului a fost evaluată la un număr total de 15582 pacienți cu diabet zaharat de tip 2 incluși în studiile clinice. Reacția adversă raportată cel mai frecvent a fost hipoglicemia, atunci când se asociază cu sulfoniluree sau insulină. **Foarte frecvente:** hipoglicemie (atunci când se utilizează împreună cu sulfoniluree sau insulină). **RA frecvente:** candidoză vaginală, vulvovaginită, balanită și alte infecții genitale; infecții ale căilor urinare, sete, prurit, erupție cutanată tranzitorie, diureză crescută, creștere a lipidelor serice. **RA mai puțin frecvente:** urticarie, depleție volemică, disurie, creșterea creatininei sanguine/scăderea ratei de filtrare glomerulară, creșterea hematocritului. **RA rare:** CAD; **cu frecvență necunoscută:** angioedem, fasceită necrozantă care afectează perineul (gangrena Fournier). **Data ultimei reînnoiri a autorizației:** 14/02/2019. **Data revizuirii textului:** 10/2019. **Jardiance® se eliberează pe bază de prescripție medicală PRF. Pentru informații suplimentare vă rugăm să consultați RCP complet, disponibil la cerere.**

TABLE OF CONTENTS

ORIGINAL RESEARCH

Changes in the Transcriptional Activity of the Lymphocyte Homing Regulatory Genes <i>Madcam1</i>, <i>Cxcr3</i>, <i>Ccr7</i> and <i>Slpr1</i> Affect Structure of the Population of T-bet⁺, Ror^γt⁺ and Foxp3⁺ cells in Mesenteric Lymph Nodes in Offspring of Rats with Experimental Gestational Diabetes Tatjana Prozorova, Oleksandr Tokarsky, Larisa Fedoniuk, Viktoriia Harbuzova, Oksana Morozova, Alexander Egorov, Alexandr Kamyshnyi	185
Metabolic Variables Influencing Body Mass Index and Overweight: A Path Analysis within a Cross-Sectional Study Marzieh Mahmoodi, Tania Dehesh, Paria Dehesh	195
Metabolic and Structural Response of Bone to Whole-Body Vibration in Obesity and Sedentary Rat Models for Osteopenia N. Kostyshyn, Yu. Kulyk, L. Kostyshyn, M. Gzhegotskyi	200
The Comparative Effect of Red Guava (<i>Psidium guajava</i> L.) with Papaya (<i>Carica papaya</i>) on Blood Glucose Level of Type 2-Diabetic Patients Zuhria Ismawanti, JB. Suparyatmo, Budiyantri Wiboworini	209
Clinical Case of Peripheral Artery Disease with Post-Thrombotic Disease in a Patient with Type 2 Diabetes Mellitus Svetlana Mykolayivna Gramatiuk, Irina Yuriivna Bagmut, Julia Viktorivna Ivanova, Michael Ivanovich Sheremet, Oleg Aleksandrovich Zarudny, Kirill Valeryevich Miasoiedov	214
Sirtuin 1, but not Osteocalcin, Correlates with Lipid Accumulation Product, Visceral Adiposity and Atherogenicity Indices in Newly Diagnosed Prediabetes-Metabolic Syndrome Patients Violet Kasabri, Abba AlBsoul-Younes, Omar Rizik, Maysa Suyagh, Kashma Omed, Govand Yassin, Sundus AlAlawi	220
The Role of Vitamin D in Treatment of Polycystic Ovary Syndrome Depending on Phenotype Halyna Suslyk, Alina Urbanovych, Orysia Lishchuk, Krystyna Kozlovska	237
Serum Testosterone and Metabolic Syndrome in Old-Aged Males: Preliminary Findings of the AHAP Cohort Study Aylar Khalilipour, Nazila Malekian, Haleh Esmaili, Seyed Reza Hosseini, Mohammadali Bayani, Ali Bijani, Fatemeh Izadpanah, Reza Ghadimi	245
Levels of pro-inflammatory cytokines (IL1β, IL18) and C-reactive protein in patients with diabetic retinopathy Marta Horecha, Lyubov Lapovets, Nataliia Lapovets, Viorika Akimova, Nataliia Bojkiv, Oksana Tsymbala, Sergii Tkachuk, Natalia Lisnianska.....	251

Determination of Serum Adiponectin Levels in Type 2 Diabetes Patients of the Saudi Population in the Al-Jouf Region	
Badr Alzahrani, Muhammad Ikram Ullah, Muhammad Atif, Ayman Ali Mohammed Alameen, Bayan Mahmoud Alkasabreh, Mohammed Al-Ruwili, Munawir Almohna, Alhmidi Alhabbad, Fares Alruwili, Abdelhadi Almejabel.....	257
Inflammatory Atherosclerosis Risk and Lipid Ratios Markers in Type 1 Diabetic Patients: The Role of Ketosis as a Metabolic State	
Walid Hassene Hamri, Mustapha Diaf, Mohamed Hadj Habib	263
Anti-inflammatory and antioxidant effects of using alpha-tocopherol in cell culture of the parotid gland under conditions similar to diabetes mellitus	
Victor Augusto Ramos Fernandes, Raphael Oliveira Ramos Franco Netto, Felipe Lovaglio Belozo, Eduardo José Caldeira.....	274
The Effect of Aerobic Training and Supplementation of Curcumin on the Glycemic Markers and Level of Myeloperoxidase Enzyme of Overweight Women	
Leila Farzad, Sepideh Dolati, Alemeh Hariri Far	281
Case Report	
Digital and Conventional Approach for the Treatment of Severe Anterior Dental Wear in a Type 2 Diabetes Patient	
Raul Rotar, Codruta Ille, Anca Jivanescu	289
Diagnosis and Management of Pheochromocytoma in a Resource-Challenged Setting: A Case Report	
Esohe Odutayo, Ijeoma Onyinye Ohuche, Muhammad Faruk Bashir1, Abel Olumuyiwa Alonge, Nuraddeen Ibrahim, Laila Matogah Adutwum, Adele Bodieu Chetcha, Mawse Amouzouvi-Sadji, Elizabeth Eberechi Oyenusi, Olumide Abiodun Elebute, Felix Makinde Alakaloko, Adesoji Oludotun Ademuyiwa, Abiola Olufunmilayo Oduwole, Ibironke Desalu	294
Review	
The Handheld Infrared Thermometry in the Diabetic Foot – Useful but Debatable Technique	
Elena-Rodica Perciun, Alexandru-Madalin Buse.....	298

Original Research

Changes in the Transcriptional Activity of the Lymphocyte Homing Regulatory Genes *Madcam1*, *Cxcr3*, *Ccr7* and *S1pr1* Affect Structure of the Population of T-bet⁺, Ror γ t⁺ and Foxp3⁺ cells in Mesenteric Lymph Nodes in Offspring of Rats with Experimental Gestational Diabetes

Tatjana Prozorova¹, Oleksandr Tokarsky^{2*}, Larisa Fedoniuk³, Viktoriia Harbuzova⁴, Oksana Morozova¹, Alexander Egorov⁵, Alexandr Kamyshnyi⁶

¹ Department of Normal Physiology, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

² Department of Medical Biochemistry, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

³ Department of Medical Biology, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

⁴ Department of Physiology and Pathophysiology, Sumy State Medical University, Sumy, Ukraine

⁵ Department of Propaedeutics and Surgical Dentistry, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

⁶ Department of Microbiology, Virology and Immunology, Zaporizhzhia State Medical University, Zaporizhzhia, Ukraine

*Correspondence to: Oleksandr Tokarsky, Ternopil National Medical University, Maidan Voli 1, Ternopil, 46001, Ukraine.

E-mail: otokarsky@tdmu.edu.ua. Phone: +38-096-410-25-36

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Abstract

Background and Aims: Maternal hyperglycemia may violate peripheral immunological tolerance (PIT) formation to pancreatic antigens; antigen administration stimulates PIT. Mesenteric lymph nodes (MLN) are transition point for recirculating lymphocytes of gastrointestinal associated lymphoid tissue (GALT) and the location for PIT induction. Homing of lymphocytes in MLN is regulated by MAdCAM-1, CXCR4 and CCR7, while S1PR1 activates T-cell exit from MLN. We aimed to evaluate mRNA expression of these genes and their effect on the distribution of Th1⁺, Th17⁺, and Treg-cells in MLN of the rat offspring with experimental gestational diabetes (EGD) with oral insulin administration. **Material and Method:** We studied one and six months old male offspring of Wistar rats with EGD, which received insulin orally for the first 14 days of life. RT-PCR method was used for *Madcam1*, *Cxcr4*, *Ccr7* and *S1pr1* mRNA expression measurements in MLN. The structure of T-bet⁺, Ror γ t⁺, Foxp3⁺ cell population was studied using histological sections of MLN, morphometric and densitometric characteristics. **Results:** An increase of CCR7 and MAdCAM-1 mRNA in offspring of animals with EGD indicated activation of the GALT immune cells, which is accompanied by intensification of lymphocytes homing, while no changes were observed in CXCR4 expression. Increased expression of S1PR1 confirms its role in diabetes. Chemokine receptors affect the activation of different Th cells subsets with their pivotal role in diabetes type 1, through violation of oral tolerance. A significant increase in Th1 and Th17 cell population was observed in the offspring of rats with EGD. **Conclusions:** The changes in mRNA expression of the lymphocyte "homing" regulators in MLN affected the distribution of Th1⁺, Th17⁺ and Treg-cells in the offspring of rats which parents had EGD. The development of oral insulin tolerance is accompanied by a decrease in the relative normalized mRNA number of *Ccr7*, *Madcam1* and *S1pr1* genes in MLN cells.

Keywords: Experimental gestational diabetes, mesenteric lymph nodes, *Madcam1*, *Cxcr4*, *Ccr7*, *S1pr1*.



Introduction

Mesenteric lymph nodes (MLN) are the main site of induction of peripheral immunological response to a variety of antigens, including pancreatic [1], as well as a major “transitional” point for the pool of recirculating lymphocytes of the gut-associated lymphoid tissue (GALT). The main regulators of lymphocyte “homing” in MLN are addressin MAdCAM-1 (mucosal addressin cell adhesion molecule 1, an adhesive protein of high endothelial venules of GALT lymphoid formations), CXCR4 chemokine receptors (CXCR4 chemokine receptor type 4) and CCR7 (chemokine receptor type 7), the ligands for which are CXCL12, CCL19, and CCL21, respectively. The chemokine-receptor interaction between the CCL19/21-CCR7 and CXCL12-CXCR4 pairs, as well as MAdCAM-1 and its ligand integrin $\alpha 4\beta 7$, regulate the transfer of most immune cells to the lymph nodes. Thus, the entry of T cells into lymph nodes is impaired in the absence of CCR7 signaling, including the *plt/plt* line mice model, lacking CCL21 and CCL19 [2]. In turn, the exit of lymphocytes from MLN is regulated by sphingosine-1-phosphate (S1P) receptors (S1PR1-S1PR5) [3], among which lymphocytes are the most actively expressing type 1 receptor, S1PR1 [4]. After the activation of immune cells in MLN, the expression of S1PR1 on their membrane increases, allowing lymphocytes to respond to changes in the S1P gradient and leave the lymph nodes [5].

The objectives of this work were to evaluate the levels of mRNA expression of MAdCAM-1, S1PR1, CXCR4, and CCR7 genes and their effect on the distribution of Th1-, Th17-, and Treg-cells in mesenteric lymph nodes in the offspring of rats with experimental gestational diabetes (EGD) and under conditions of oral insulin administration.

Material and Methods

Study design

The male Wistar rats (N=120) were divided into 6 experimental groups, 20 rats per each

group: the offspring of intact rats, 1-month-old (group 1) and 6 months old (group 2), female parents of which were administered 0.5 ml of 0.1 M citrate buffer (pH = 4.5) intraperitoneally on the 15th day after pregnancy; the offspring of rats with experimental gestational diabetes (EGD) at 1 month (group 3) and 6 months age (group 4), female parents of which were once injected streptozotocin at a dose of 45 mg/kg on the 15th day of pregnancy; offspring of rats with EGD at 1 month of age (group 5), female parents of which were administered oral short-acting human insulin (ACTRAPID® HM, NOVO NORDISK, Denmark) at a dose of 30 IU (1.05 mg, 1 IU corresponds to 35 μ g of anhydrous human insulin) orally during the first 14 days of life; offspring of rats with EGD at 6 months of age (group 6), female parents of which were given 30 IU insulin orally during the first 14 days of life.

Molecular genetics experiments

Molecular genetics experiments using the real-time reverse transcription-polymerase chain reaction (RT-PCR) method were performed on isolated MLN of experimental rats. The tissue was placed in a Bouin's fixative solution (15 parts of the saturated aqueous picric acid solution, 5 parts of formaldehyde, 1 part of acetic acid), followed by tissue dehydration using ascending concentrations of ethanol, and then placed in paraffin blocks. The experiments were conducted on archival material not older than 2 years. RNA was obtained from histological sections with a thickness of 15 μ m, by their deparaffinization in xylene followed by rehydration in descending concentrations of ethanol (100%, 96%, 70%). Total RNA was isolated using a Trizol RNA Prep 100 kit (Isogen Lab., LTD, Russian Federation) containing Trizol reagent (a lysing reagent comprising guanidine thiocyanate as denaturing agent and phenol with pH = 4.0) and ExtraGene E (suspension of ion exchange mixture). The RNA was isolated according to the manufacturer's instructions.

cDNA synthesis was carried out by reverse transcription reaction using a commercial kit OT-1 (Synthol, Russian Federation). The reaction

Table 1: List and description of genes and corresponding primers used during experiments

Gene	Primer	Tm, °C	Product length (bp)	Exon junction
Cxcr4	F = TGCCATGGAAATATACACTTCGG R = TCCAGACCCTACTTCTTCGGA	58.87 59.64	52	81/82
Ccr7	F = CTGGTCATTTTCCAGGTGTGC R = TGGTGTCTCGCCGATGTAG	59.73 59.83	67	138/139
Madcam1	F = CCAAAGTGGTGCTGACACAT R = TCTGGCTCTGTAGGACTGGAA	58.68 59.92	51	662/663
Slpr1	F = CGGATCGCGCGGTGTAG R = GAAACAGCAGCCTCGCTCAA	60.73 61.23	70	61/62
GAPDH	F = GCCTGGAGAAACCTGCCAAG R = GCCTGCTTCACCACCTTCT	61 60	52	825/826

mixture with a total volume of 25 µl contained 1 µl of Random-6 primer, 2 µl of total RNA, 8.5 µl of deionized H₂O, 12.5 µl of 2.5x reaction mixture, and 1 µl of MMLV-reverse transcriptase. Reverse transcription was carried out at 45°C for 45 min, followed by MMLV-RT heat inactivation (92°C, 5 min), as suggested by the manufacturer.

The CFX96™ Real-Time PCR Detection Systems Amplifier (Bio-Rad Laboratories, Inc., USA) and Maxima SYBR Green/ROX qPCR MasterMix (2X) reagent kit (ThermoScientific, USA) were used to determine expression levels of mRNAs of the investigated genes, namely, Madcam1 (NM_019317.1), Cxcr4 (NM_022205.3), Slpr1 (NM_017301.2), Ccr7 (NM_199489.4). The final reaction mixture for amplification included SYBR Green dye, Maxima HotStartTaq DNA Polymerase, 0.2 µl of forward and reverse specific primers, 1 µl of the template (cDNA). The reaction mixture was brought to a total volume of 25 µl with the addition of deionized H₂O. Gene identification numbers (NM_019317.1, NM_022205.3, NM_017301.2, NM_199489.4) were derived from the US National Library of Medicine, National Center of Biotechnology Information website (<https://www.ncbi.nlm.nih.gov/>). Specific primer pairs (forward and reverse) for amplification of the investigated and reference genes were selected using the PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and manufactured by Metabion, Germany (Table 1). After the initial denaturation for 10 min at 95°C, the amplification consisted of 45 cycles and each cycle was performed under the following conditions: denaturation - 95°C for 15 sec, annealing

-59-61°C for 30-60 sec, elongation -72°C for 30 sec. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a reference gene to determine the relative values in changes in the expression level of the studied genes and normalize results. The relative normalized number of cDNA target genes was determined by the $\Delta\Delta C_t$ method.

Immunohistochemistry

The population structure of T-bet + - (Th1), Ror γ t + - (Th17), and Foxp3 + - (Treg) lymphocytes was studied by analyzing serial histological sections of MLN and their morphometric and densitometric characteristics. MICROM HR-360 rotary microtome (Microm, Germany) was used to make 5 micron serial MLN sections. The histological sections were dewaxed in xylene, followed by rehydration in decreasing concentrations of ethanol (100%, 96%, 70%), then washed in 0.1 M phosphate buffer (pH = 7.4), and treated with primary rabbit polyclonal antibodies (PRPA) to T-bet, Ror γ t or Foxp3 (Santa Cruz Biotechnology, USA, sc-1550-R) for 18 hours in a humid chamber at 4°C. After washing off the excess of primary antibodies in 0.1 M phosphate buffer, histological sections were incubated for 60 min at 37°C with secondary antibodies to the full rabbit IgG molecule, conjugated to FITC (Santa Cruz Biotechnology, USA). After incubation, all sections were washed with 0.1 M phosphate buffer and placed in a mixture of glycerol and phosphate buffer (1:9) for further fluorescence microscopy.

Images were taken with a Primo Star microscope (ZEISS, Germany) in a 390 nm ultraviolet excitation spectrum (FITC) using a high-sensitivity Axio Cam 5c camera (ZEISS, Germany) and processed with a software package for obtaining, archiving and preparing images (Axio Vision 4.7). The processed histological sections were examined using a computer program ImageJ (National Institute of Health, USA). Image regions with statistically significant fluorescence were determined in the automatic mode using specific software. The morphometric and densitometric characteristics of immunopositive cells were calculated. The absolute number of immunopositive cells of different classes as the number of cells per 1 mm² slice area and relative (%) density of their distribution in the studied areas of MLN histological sections were determined.

Statistical analysis

For molecular genetics experiments, three sets of histological sections from MLN of each rat were used for RNA extraction. Following cDNA synthesis from each set, PCR amplification reactions were performed on individual samples in three repetitions. Negative controls were included in all experiments, including the absence of MMLV-RT in cDNA synthesis, absence of mRNA in the matrix of cDNA synthesis, and absence of cDNA matrix in the real-time PCR reaction. Student's t-test ($p < 0.05$) was used to determine if the means of two sets of data are significantly different from each other (control vs. EGD, EGD vs. EGD+insulin). Statistical analysis of PCR data was performed using the CFX Manager™ software (Bio-Rad, USA) with a statistical significance threshold set at $p < 0.05$.

For immunochemistry experiments, three histological sections were used from the cortical plateau and medullar cords of MLN of each rat to determine the absolute number of immunopositive cells (number of cells per 1 mm² slice area) and relative (%) density of their distribution from five studied areas of MLN histological sections. Student's t-test ($p < 0.05$) was used to determine if the means of two sets of data are significantly different from each other (control vs.

EGD, EGD vs. EGD+insulin). Statistical analysis of immunochemistry data was performed using Statistica ver. 10 (StatSoft, Inc., Tulsa, OK, USA).

Results

The results of the Ccr7 gene expression study in MLN tissues showed that there was a significant increase in mRNA content of this chemokine receptor in offspring of EGD rats, with a 26.8-fold increase in 1-month and a 21-fold increase in 6-month-old offspring compared with the control group of animals of the corresponding age ($p < 0.05$, Figure 1 A, B). As for the expression of CXCR4 receptor mRNA, its content did not change significantly in both age groups compared to control ($p > 0.05$, Figure 1 C, D). The relative normalized addressin MAdCAM-1 mRNA expression increased 2.4 times and 2.3 times in the age group of 1 month and 6 months offspring of EGD rats, respectively ($p < 0.05$, Figure 1 E, F). Regarding Slpr1 gene expression, the content of S1PR1 mRNA increased 3.6 times ($p < 0.05$) in the 1-month group of offspring rats with EGD; in the 6-months group, a 5.0-fold increase was observed compared to the control group ($p < 0.05$, Figure 1 G, H).

Expression of mRNA of the Ccr7 gene in 1-month-old offspring of EGD rats after oral administration of insulin during the first 14 days of life decreased by 81% compared to offspring of untreated EGD rats of the same age as the control group ($p < 0.05$). However, no significant difference was observed between corresponding groups at 6-months age ($p > 0.05$) (Figure 2 A, B). Cxcr4 gene expression did not change significantly in both age groups while comparing the offspring of EGD rats treated with insulin and the offspring of untreated EGD rats (Figure 2 C, D). Contrary, the MAdCAM-1 mRNA content decreased by 95% at 1 month of age as a result of insulin administration ($p < 0.05$), whereas in 6-month-old rats, a 3.9-fold increase was noticed ($p < 0.05$) (Figure 2 E, F). The mRNA content of the Slpr1 gene decreased by 65% and 95% for the 1-month and 6-months offspring of EGD rats treated orally with insulin ($p < 0.05$) (Figure 2 G, H).

The changes detected in the level of expression of the regulators of lymphocyte

“homing” in MLN influenced the distribution of individual subpopulations of T-helper cells. For example, the total density of T-bet⁺-cells in the cortical plateau in the 1-month-old offspring of animals with EGD compared to the control group increased 2-fold; in 6-months rats, we observed an increase of 56%. Regarding medullary cords (chordae medullares), this index increased by 2.6 and 2.8 times in 1-month and 6-months rats, in offspring of EGD rats against control rats, respectively ($p < 0.05$). In the group of animals receiving oral insulin, a decrease in the total density of T-bet⁺ lymphocytes in the cortical plateau was observed by 33% ($p < 0.05$) at 1-month and by 41% ($p < 0.05$) in 6-month-old rats. In the medullary cords, the total amount of Th1 after insulin administration decreased only in animals aged 6 months - by 44% ($p < 0.05$).

Analysis of the distribution of Ror γ ⁺-cells in MLN tissue of rats showed that in the 1-month-old offspring of rats with EGD, the total Th17 cell density in cortical plateau was significantly higher - by 48% ($p < 0.05$) - than the control group of offspring from intact rats. In 6 months-old animals, the number of Ror γ ⁺ lymphocytes in this zone increased by 2.4 times. In medullary cords, the total Th17 density in the 1-month-old offspring of EGD animals increased by 51% ($p < 0.05$); in 6-month-old rats - by 2.4 times ($p < 0.05$), compared to the offspring of intact animals of corresponding age group. After insulin administration, the total lymphocyte density of Ror γ ⁺ lymphocytes in the cortical plateau of 1-month-old animals did not change significantly, and at 6 months of age, it decreased ($p < 0.05$). In the medullary cords, the number of Ror γ ⁺ -cells decreased by 52% ($p < 0.05$) in the 1-month-old offspring and by 46% ($p < 0.05$) in the 6-month-old offspring.

The number of Foxp3⁺ lymphocytes in the cortical plateau of the offspring of rats with EGD was lower by 57% in 1-month-old animals ($p < 0.05$), and lower by 54% ($p < 0.05$) in 6-months old animals, compared with the control group. A decrease in T-regulatory cells by 76% and 64% ($p < 0.05$) was also observed in medullary cords. In rats treated with insulin, the following results were found: the total density of Foxp3⁺ cells in 1-month-old rats in the cortical plateau did not

change significantly. Still, it increased 2.2-fold in 6 months-old animals ($p < 0.05$). The number of Treg increased in the medullary cords by 2.3 - 2.6 times ($p < 0.05$).

Discussion

Earlier, we showed that under conditions of diabetes, there are changes in the transcriptional activity of some genes and the subpopulation composition of T-lymphocytes [6-8]. CCR7-mediated signals affect T-cell homeostasis in lymph nodes at different levels, as well as the activation and polarization of different T-helper subpopulations [9]. Thus, CCR7 plays an essential role in the function of T-regulatory cells, while performing a dual role: for Treg-induced expression, CCR7 expression is required for their efficient priming in lymph nodes [10]; while effector Treg apparently uses CCR7 to exit tissues and, therefore, CCR7 limits their excessive accumulation in peripheral tissues [11]. Although the total number of Treg in the thymus in CCR7 knockout mice and wild-type mice is the same, their suppressor functions in the periphery are impaired due to their inability to enter the lymph nodes and position themselves within the T-cell zones of LN [12]. A recent study by Mackley et al. showed that migration of a group of congenital ROR γ -lymphocytes from the intestine into MLN is also CCR7-dependent [13], and such cells intentionally migrate precisely to MLN and not to other groups of LN. CCR7^{-/-} mice are more susceptible to infections caused by *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Leishmania donovani* and *Toxoplasma gondii* [14], showing an increase in lymphocytic infiltration of peripheral tissue and pancreatic islets, disturbances in central and peripheral tolerance to their own antigens and defects in the functioning of Treg cells, leading to the development of multiple organ autoimmune reactions [15]. This is accompanied by an increase in the titer of circulating auto-antibodies to a large number of tissue-specific antigens and the formation of ectopic tertiary lymphoid structures on the mucous membranes [16]. Shan et al. established the important role of CCR7 in engaging T cells in pancreatic islets in

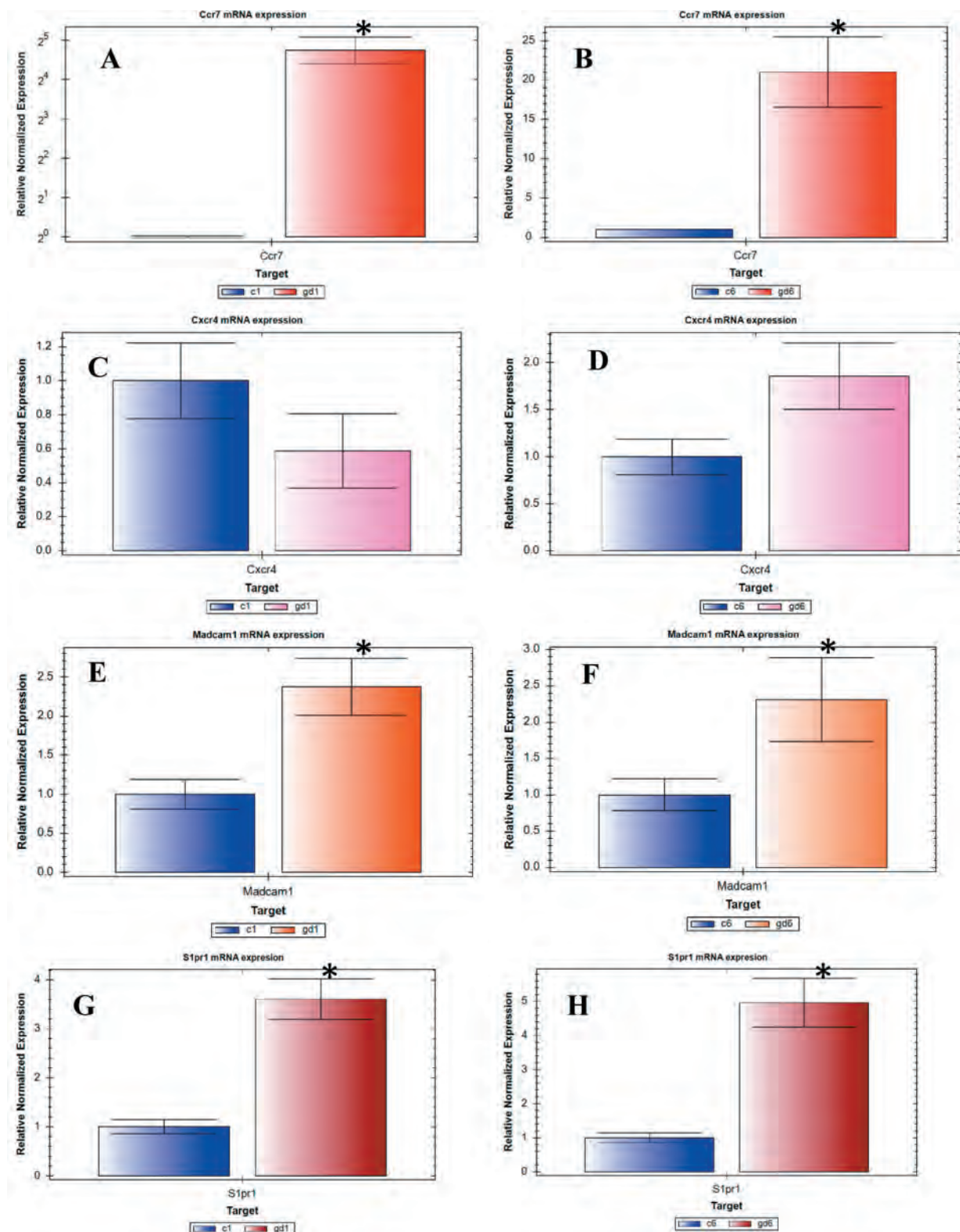


Figure 1: Relative normalized mRNA number of genes Ccr7 (A, B), Cxcr4 (C, D), Madcam1 (E, F) and S1pr1 (G, H) in the cells of MLN tissue. Normalization performed by $\Delta\Delta$ Ct method with GAPDH as reference gene (c1 – 1-month old control offspring from intact rats, c6 – 6-months old control offspring from intact rats, gd1 – 1-month old offspring from EGD rats, gd6 – 6-months old offspring from EGD rats). Asterisk (*) indicates a significant difference ($p < 0.05$).

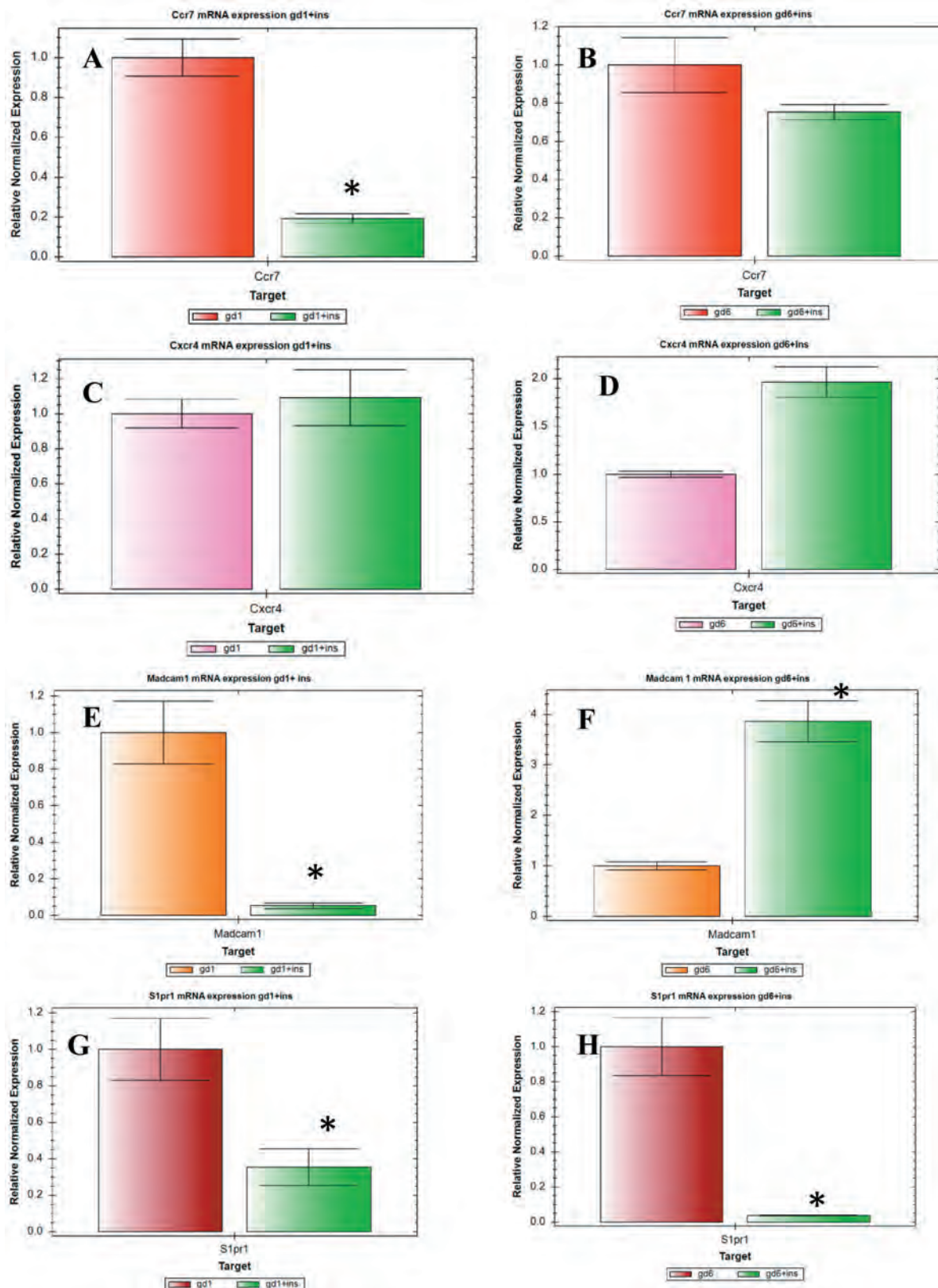


Figure 2: Relative normalized mRNA number of genes Ccr7 (A, B), Cxcr4 (C, D), Madcam1 (E, F) and Slpr1 (G, H) in the cells of MLN tissue. Normalization performed by $\Delta\Delta$ Ct method with reference gene of GAPDH. (gd1 – 1-month old control offspring from EGD rats, gd6 – 6-months old control offspring from EGD rats, gd1+ins – 1-month old offspring from EGD rats given insulin orally, gd6+ins – 6-months old offspring from EGD rats given insulin). Asterisk (*) indicates a significant difference (p < 0.05).

NOD-mice [17], using RT-PCR showed intensive expression of CCR7 mRNA and its CCL19 and CCL21 ligands in inflamed, but not intact islets. Turning off CCR7 signaling blocked the migration of about 75% of T cells from the blood to the pancreatic islets, but this did not affect the migration of B lymphocytes to the islets. The received results indicate that CCR7 and its ligands play an essential role in T-cell entrance in the islets and, thus, in the pathogenesis of type I diabetes mellitus. Some studies have demonstrated the role of CCR7 signaling in the formation of oral tolerance to various antigens. However, oral tolerance cannot be induced in CCR7-deficient mice due to impaired migration of dendritic cells, which carry those antigens from the intestinal mucosa of the intestine into the MLN [18].

The increase in the level of mRNA of another of the regulators of “homing” of lymphocytes - MAdCAM-1 in MLN is confirmed by the results of previous research in which it is shown that RNA transcripts of MAdCAM-1 are well expressed in the endothelium of both lymphoid and non-lymphoid tissues, including small and large intestines, mesenteric and pancreatic lymph nodes, spleen [19]. Characteristically, this expression is increased in response to the action of proinflammatory cytokines, in animal models of inflammatory bowel disease, and patients with Crohn's disease and ulcerative colitis [20]. MAdCAM-1 is exclusively expressed in high endothelial venules MLN, but not peripheral LN [21]. There are no data on the expression of MAdCAM-1 in MLN in offspring of parent rats having EGD. Only lymphocyte adhesion, regulated by the $\alpha 4\beta 7$ integrin/MAdCAM-1 system, is known to be essential for B-cell migration into pancreatic lymph nodes (PLN) in non-obese diabetic (NOD) mice. Thus, B-lymphocytes from PLN of 3 to 4-week old NOD mice are characterized by high expression of integrin $\alpha 4$, LFA-1, and intermediate level of integrin $\beta 7$. In-vivo studies showed that B cells migrated from the bloodstream to PLN more efficiently than other peripheral LN. Also, antibodies to MAdCAM-1 and integrin $\alpha 4\beta 7$ inhibited the B lymphocyte migration into PLN by more than 90% [22]. Single intraperitoneal injection of anti-MAdCAM-1 antibodies to 3-week-old NOD

mice significantly slowed B-lymphocyte entry into the PLN for at least 2 weeks. These results demonstrate that the $\alpha 4\beta 7$ integrin/MAdCAM-1 adhesion pathway plays a vital role in the migration of B lymphocytes into PLN in NOD mice and might be a potential target for T1DM therapy. The role of MAdCAM-1 in the development of type 1 diabetes mellitus was shown by other researchers as well. Thus, CD4 + cells reactive to pancreatic islets are able to initiate the development of type 1 diabetes when transferred to newborn NOD mice, but not in adult animals. These differences are due to unequal levels of MAdCAM-1 expression in pancreatic islets in newborn and adult mice [23]. Besides, blockade of this adhesion interferes with the induction of diabetes in newborn mice.

In the final stage of lymphocyte homing inside LN, another chemokine receptor, namely CXCR4, also known as fusin or CD184, plays an important role. Specifically, it binds to the ligand - the stromal-derived factor (SDF-1) molecule. Under normal physiological conditions, SDF-1 is produced by stromal cells of many tissues, and CXCR4 is expressed on the surface of CD4+ T-cells, dendritic cells and B lymphocytes. We did not detect changes in CXCR4 mRNA levels in MLN in the offspring of rats with EGD, although other studies have shown that the CXCR4/CXCL12 signaling pathway protects NOD mice from autoimmune diabetes [24]. CXCR4 mRNA levels were increased in the PLN of 4-week NOD mice compared with Balb/C mice [25]. A significant decrease in the level of CXCR4 expression was observed in 12 weeks-old animals regarding both mRNA expression and protein levels, while their content in pancreatic islets increased. The introduction of AMD3100, a CXCR4 antagonist, reduced the number of CXCR4+ and SDF1+ positive cells in inflamed islets and provided protective effects against autoimmune diabetes. Simultaneously, the transcriptional level of CXCL12, a Cxcr4 ligand, is significantly increased in the bone marrow of NOD mice compared to Balb/c and C57BL/6 mice. The introduction of AMD3100 into NOD mice mobilizes regulatory T cells and hematopoietic bone marrow stem cells to the periphery and suppresses insulinitis and slows the onset of diabetes [26]. Transgenic mice,

which are overexpressing SDF-1, are resistant to streptozocin-induced β -cell apoptosis and diabetes, whereas MIN6, another CXCR4 antagonist, causes their apoptosis, increases the production of ROS, reduces the expression of anti-apoptotic protein BCL-2 and phosphorylation of the proapoptotic BAD protein [27].

Inhibition of S1PR on T or B lymphocytes (FTY720, fingolimod) renders these cells insensitive to a change in the S1P concentration gradient between lymph nodes and biological fluids; thus, provides CCR7 receptor-mediated lymphocyte retention in lymph nodes [28]. FTY720 has demonstrated its effectiveness in several models of immunological diseases, including rheumatoid arthritis, myasthenia gravis, multiple sclerosis, and type 1 diabetes mellitus [29]. The increased level of S1PR1 mRNA expression in MLN lymphocytes, which we found, may play an important role in the development of diabetes. Thus, the cessation of lymphocytes exits from PLN by the S1P agonist FTY720 receptors in NOD mice prevents further progression of diabetes even during significant insulinitis [30]; after its use, no increase in expression levels of cytotoxic molecules of granzyme B, interferon γ , and CD107a in islet CD8-lymphocytes was observed [31]. Mesenteric lymphadenectomy significantly reduces the size of the population of Th17 lymphocytes in the intestine. The introduction of FTY720 induces the accumulation of $\alpha 4\beta 7$ + IL-17A+ proliferating cells in MLN [32].

Conclusion

The changes we detected in the level of expression of the regulators of lymphocyte “homing” in MLN affect the distribution of Th1-, Th17- and Treg-cells in the offspring of rats who had EGD. The development of oral insulin tolerance is accompanied by a decrease in the relative normalized mRNA number of *Ccr7*, *Madcam1* and *S1pr1* genes in MLN cells, total Ror γ + and T-bet+ lymphocyte density.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Metabolic Variables Influencing Body Mass Index and Overweight: A Path Analysis within a Cross-Sectional Study

Marzieh Mahmoodi¹, Tania Dehesh^{2*}, Paria Dehesh³

¹ Department of Biostatistics, School of Health and Nutrition, Bushehr University of Medical Sciences, Bushehr, Iran

² Department of Biostatistics and Epidemiology, Kerman University of Medical Sciences, Kerman, Iran

³ Department of Epidemiology, Iran University of Medical Sciences, Tehran, Iran

*Correspondence to: Tania Dehesh, Haft Bagh Alavi, Kerman University of Medical Sciences, Iran, Kerman, Postal code: 7617647633.

E-mail: Tania_dehesh@yahoo.com. Phone: +98-34-31325069, Fax: +98-34-31325070

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Abstract

Introduction: This study aims to explore the most responsible, influential metabolic variables of Body Mass Index (BMI) and investigating their direct and indirect effect on path analysis. **Material and Methods:** 350 adults aged 18 years and older without any chronic diseases who came for an annual blood check-up were included in this study. Thyroid-Stimulating Hormone (TSH), Fasting Blood Sugar (FBS) and Thyroid-Stimulating Hormone (TSH) levels were measured using standard laboratory methods. Path analysis was carried out to examine the triglyceride (TG) mediator role in the relationship between age, FBS, and TSH on BMI. **Results:** TSH has a significant indirect effect on BMI ($P=0.017$). Age has both direct and indirect effects on BMI ($P=0.003$). Age and TSH have a direct impact on TG levels ($p < 0.05$), and the mediator of TG has a direct effect on BMI ($P=0.001$). **Conclusions:** Controlling some biomedical factors leads to controlling obesity as one of the main concerns in public health. Unexpectedly, TSH even had an indirect effect on BMI through TG. Also, because BMI is indirectly affected by age with the mediator of TG, controlling triglyceride along with increasing age, becomes more important.

Keywords: Biomedical factors, obesity, body mass index, triglyceride (TG), fasting blood sugar (FBS), thyroid-stimulating hormone (TSH).

Introduction

Obesity is one of the most significant public health problems globally. Overweight and obesity increase the risk of various diseases, especially heart disease, type 2 diabetes, sleep apnea blockers, certain types of cancer, bone turnover, and asthma [1-5]. As a result, one of the factors that reduce average life expectancy is obesity [1]. According to available statistics, the death rate of obesity is higher than the total mortality rate due to four common cancers (lung, breast, intestinal and prostate). Obesity has been known as one of the most preventable causes of death worldwide.

On average, overweight and obesity reduce life expectancy by about 6 to 7 years [6].

Based on the World Health Organization (WHO) report in 2016, about 39% of adults above 18 years old were overweight, and 13% (11% of men and 15% of women) of them were obese [7].

In Iran, the prevalence of obesity was estimated at 21.7% in populations aged 18 years and over and 6.1% in populations below 18 years old [8].

Using BMI is the most common way of estimating overweight and obesity. In Asia, the risk of adverse health effects starts to increase progressively by increasing BMI from a value between 22 to 25 kg/m² [9].



Some factors can affect BMI and lead to overweight or obesity. These include individual elements such as diet, lack of exercise, environmental factors, and genetics. The accumulation of fat or triglycerides (TG) is essentially the only metabolic variables for overweighing the body [6]. However, many previous studies have shown that both TG and age are closely and independently related to BMI [10, 11]. These studies used an analysis that examines the direct effect of these variables on BMI and overweight.

Other studies have shown that some metabolic variables can affect the TG level, such as FBS and TSH [12-14].

However, none of these studies have considered all these variables together. Also, there were limitations in understanding the direct and indirect effects of these factors on BMI.

To overcome these limitations, this study aims to evaluate how the variables are related to BMI and overweight, which have been identified by other studies. To this end, we carried out a path analysis to examine the TG mediator's role in the relationship between age, FBS, and TSH on BMI.

Material and Methods

This cross-sectional study was carried out on the population referred to the best clinic in Kerman, southern Iran, for an annual check-up from March to September 2018. Three hundred fifty adults aged 18 years and older without any chronic diseases were included in the study. The subjects who had obesity of endocrine origin and pregnant and lactating women were excluded from the study.

After receiving informed consent and approval from the ethics committee of Kermand University (reference number: IR.KMU.REC.1397.174), the people were enrolled for the study.

Blood samples were collected from participants that practiced fasting. Serum samples were centrifuged at 3000 rpm for 5 min and stored at -20°C until analysis. Also, TG, FBS and TSH levels were measured using standard laboratory methods.

Weight and height were measured using standardized techniques and equipment. BMI

was calculated as weight in kilograms and was divided by the square of the height in meters. BMI provides the most useful measure for overweight and obesity. The same technique was applied for both adult men and women and all ages.

For adults, the WHO defines overweight as a BMI greater than or equal to 25 and obesity as a BMI greater than or equal to 30[7].

Statistical analysis

The data are summarized as frequencies and percentages for categorical variables. Continuous variables are reported as mean $\pm$ Standard Deviation (SD).

Pearson's correlation coefficients were calculated for the evaluation of the relationship between study variables. Afterward, path analysis was used to identify factors that are influencing BMI. For the interpretation of effects, the path analysis or causal modeling was developed as a method of decomposing correlations into different parts. The method is applied to describe the direct and indirect correlations among a set of variables. Exogenous independent variables were age, TG, FBS and TSH levels. The causal assumptions were displayed in a path diagram in which the relationships were specified between variables and the causality direction.

SPSS version 20.0 was used for the descriptive statistical analysis, and AMOS version 20.0 was used for path analysis. The significance level of 0.05 was used for tests.

Results

Table 1 describes the characteristics of the 350 participants in this study. Among the 350 participants, 169 (48.3%) were male, and 181 (51.7%) were female.

A mean of 43.3 and a standard deviation of 13.94 was obtained for the age variable. There was no significant difference between the age of women and men ($P=0.490$).

The mean ($\pm$ SD) BMI of the people was equal to 26.85 ($\pm$ 5.19) kg/m². It appeared that the mean BMI of women was significantly higher

Table 1: Subject characteristics and lab values*

	Total (n = 350)	Women (n = 181)	Men (n = 169)	P-value
Age	43.3 ± 13.94	43.80 ± 13.56	42.76 ± 14.36	0.490
Education				
Under diploma	188(53.7)	104(57.5)	84(49.7)	0.128
Diploma	100(28.6)	52(28.7)	48(28.4)	
Associate Degree	19(5.4)	5(2.8)	17(10.1)	
Bachelor	34(9.7)	17(9.4)	17(10.1)	
M.S	9(2.6)	3(1.7)	6(3.6)	
BMI	26.85 ± 5.19	28.29 ± 5.53	25.31 ± 4.32	< 0.001
Underweight (<18.5)	14(4)	3(1.7)	11(6.5)	< 0.001
Normal weight (18.5-24.9)	125(35.7)	52(28.7)	73(43.2)	
Overweight (25-29.9)	123(35.1)	66(36.5)	57(33.7)	
Obesity class 1 (30-34.9)	70(20)	44(24.3)	26(15.4)	
Obesity class 2 (35-39.9)	12(3.4)	10(5.5)	2(1.2)	
Morbidity obesity (>40)	6(1.7)	6(3.3)	-	
FBS	109.08 ± 35.49	108.29 ± 6.26	109.93 ± 34.73	0.665
TSH	2.63 ± 2.06	2.81 ± 2.11	2.44 ± 2.01	0.094
TG	156.73 ± 97.12	153.04 ± 95.87	160.69 ± 98.56	0.462

*Age, BMI, FBS, TSH and TG were expressed as mean ± SD. Education and BMI categories were expressed as frequency (%).

than that of men ($P > 0.001$). As shown, more than half of the people (60.3%) had a BMI > 25 and they were considered overweight or obese. The number of people with normal weight (BMI 18.5-24.9) was 125, and just a very small number (4%) were assumed to be underweight.

The mean ($\pm$ SD) FBS was equal to 109.08 ($\pm$ 35.49) mg/dL, and mean ($\pm$ SD) TSH was equal to 2.63 ($\pm$ 2.06) U/ml. The mean $\pm$ SD TG of males was higher than that of females.

The mean $\pm$ SD TG of male and female participants was equal to 153.04 95.87 and 160.69 98.56, respectively. There was no significant difference in biomedical factors (FBS, TSH and TG) between males and females ($P = 0.05$).

Path analysis results

The path analysis was developed to examine the direct and indirect paths of variables that affect BMI. The findings of previous studies were used to define the paths of each of these variables

in the hypothetical model and to identify how they affect BMI. Figure 1 illustrates a diagram of the basic model with paths created between the different study variables.

Goodness of fit

In order to assess how well this model fits the data, we used several statistical indices to check the data fit degree. The most common indices of fit are the chi-square (χ^2), comparative fit index (CFI), the Normed Fit Index (NFI), the goodness of fit index (GFI), and the root mean square error of approximation (RMSEA). The analysis showed that all indices belonged to an appropriate range, and all indicated a good fit. $\chi^2 = 1.856$ (df = 2), $p = 0.392$, GFI = 0.998, NFI = 0.979, CFI = 1.000, RMSEA = .000 (Table 2).

Table 3 summarizes the estimates of standardized direct, indirect and total effects of FBS, TSH, TG and age on BMI. TSH has a significant indirect effect ($P = 0.017$) and age has both direct

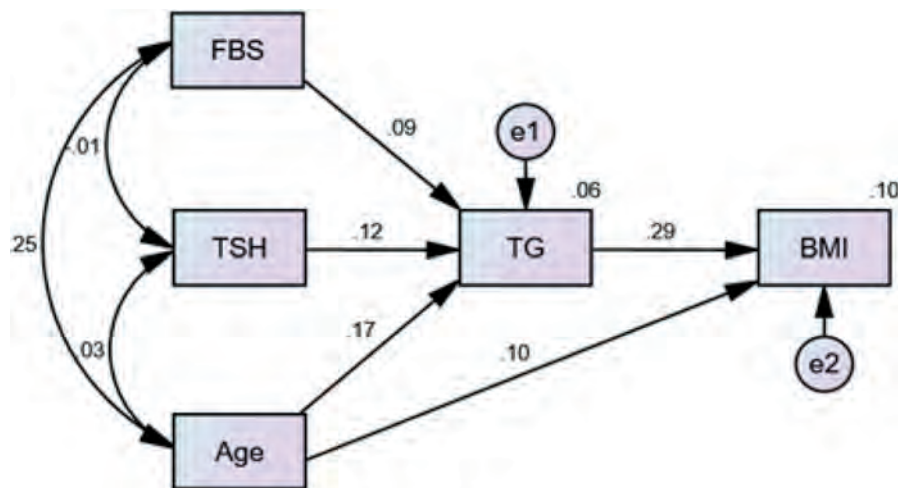


Figure 1: The path diagram of BMI effective predictors.

Table 2: Goodness of fit indices.

CMNI (χ^2)	DF	P-value	GFI	NFI	CFI	RMSEA	CMNI/DF
1.856	2	0.392	0.998	0.979	1	0.000	0.928

Table 3: The direct, indirect and total effects of exogenous on endogenous variables.

	Direct			Indirect			Total		
	effect	C.I	P-value	effect	C.I	P-value	effect	C.I	P-value
FBS → TG	0.086	(-0.191, 0.021)	0.109				0.086	(-0.192, 0.021)	0.109
Age → TG	0.175	(0.782, 0.258)	0.002				0.175	(0.781, 0.258)	0.002
TSH → TG	0.124	(0.015, 0.227)	0.024				0.124	(0.015, 0.227)	0.024
TG → BMI	0.288	(0.184, 0.409)	0.001				0.288	(0.184, 0.409)	0.001
Age → BMI	0.099	(0.003, 0.193)	0.041	0.051	(0.019, 0.089)	0.001	0.149	(0.051, 0.241)	0.003
FBS → BMI				0.025	(-0.004, 0.061)	0.093	0.025	(-0.004, 0.061)	0.093
TSH → BMI				0.036	(0.005, 0.082)	0.017	0.036	(0.005, 0.082)	0.017

and indirect effects on BMI ($P=0.003$). Age and TSH have a direct effect on the TG ($p<0.05$) and the mediator of TG has a direct effect on BMI ($P=0.001$).

Discussion

In this study, BMI and laboratory parameters were evaluated and data were compared to find associations between these parameters. To the best of our knowledge, this is the first study using path analysis for determining the biomedical parameters of BMI. Some studies have been conducted to test the effect of each of the biochemical factors on BMI. However, the issue of

whether the relationship is direct or indirect (or both) has not been considered [10-14].

According to the descriptive results of this study, more than half of the people (60.3%) were considered overweight or obese. This rate of obesity is higher than the global obesity rate of adults aged 18 years and above [7], and it should be considered a severe health problem in the Kermanian population.

The mean value of BMI of women was higher than men, and most people with overweight and obesity were women. The present study results are very similar to the results reported from other studies on the population of the north of Iran. In the study of Hajian-Tilaki,

the overall prevalence of overweight and obesity was 53.6% (61.3% of women and 46.1% of men) [15]. In another study on the population of Azarbayejan of Iran, the prevalence of overweight was 36.5% (38.6% of men and 34.8% of women) and the prevalence of obesity was 33.3% (25.4% of men and 40.0% of women) [16]. In a nationwide survey of the Iranian population aged 15-65, the prevalence of overweight and obesity was 42.8% in men and 57% in women [17].

In this study, Path analysis results showed age and TSH have an indirect effect on BMI with the mediator of TG. Therefore, the TSH factor plays an essential role in reducing BMI.

Controlling TSH leads to BMI control directly. On the other hand, TSH reduces BMI indirectly by reducing TG. The previous studies found just a direct relationship. Nyrdnes showed that serum TSH is positively associated with BMI [18]. The study of Shaoba showed a direct positive association between TSH levels and BMI [19] and the study of Bétry confirmed the relationship between TSH and BMI [20].

Conclusion

These findings highlight the necessity of designing programmes to control the biomedical factors associated with obesity, especially TSH. Also, because BMI and the mediator of TG are indirectly affected by age, controlling triglyceride levels is necessary at an older age.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Metabolic and Structural Response of Bone to Whole-Body Vibration in Obesity and Sedentary Rat Models for Osteopenia

N. Kostyshyn^{1*}, Yu. Kulyk², L. Kostyshyn³, M. Gzhegotskyi¹

¹ Department of Normal Physiology, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

² Department of Metal Physics, Ivan Franko National University, Lviv, Ukraine

³ Department of Toxicological and Analytical Chemistry, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

*Correspondence to: Kostyshyn N.M., PhD, Department of Normal Physiology, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine. E-mail: kostyshyn.nm@gmail.com <https://orcid.org/0000-0003-0341-5853>

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Abstract

Introduction: Several studies indicate that obesity is associated with increased bone mass due to excessive mechanical stress. However, these data are controversial, and the risk of fractures in this group is high. This study aimed to investigate the effect of high-frequency whole-body vibration on the process of bone remodeling in rats with a sedentary lifestyle and obesity. **Material and Methods:** To assess bone metabolism in rat blood plasma, we determined the parathyroid hormone and calcitonin levels to quantify the loss of the bone mineral component, the x-ray diffraction method was used. **Results:** Using X-ray diffraction, we observed the increase of the crystalline phase volume fraction in rats with whole-body vibration (WBV), and the decrease of the mineral component in the first experimental group – rats with sedentary lifestyle and obesity. Besides, the first experimental group showed a higher concentration of the parathyroid hormone in the serum and lower calcitonin levels. It is worth noting that the experimental animals with WBV showed a decrease in fat mass and leptin concentrations in blood serum. **Conclusions:** The results indicate that obesity has a negative impact on mineral bone mass. Mechanical stimulation may serve as a promising alternative to medication therapy for osteopenia.

Keywords: Bone remodeling, bone mineral density, osteoporosis, X-ray diffraction.

Abbreviations: BMD – bone mineral density; HCD – High-calorie diet; LMC – Limited mobility condition, PTH – parathyroid hormone, RANKL/RANK/OPG – receptor activator of nuclear factor (NF)-kB-ligand / receptor activator of NF-kB / receptor osteoprotegerin; WBV – whole body vibration.

Introduction

Every year we witness an increase in the number of people with pathological disorders of the musculoskeletal system. Therefore, the study of the influence of various factors on bone metabolism is relevant. Sedentary lifestyle and obesity are among the factors that might influence bone mineral density. Calcium homeostasis is strictly regulated by several factors, such as the RANKL/RANK/OPG cytokine system, estrogens, vitamin D3, parathyroid hormone, and calcitonin. It also depends on one's digestive system absorption and

lifestyle. Since the bone is a mechanosensitive tissue, its condition depends both on the mentioned factors and exogenous influences, e.g., vibration oscillations. Several studies indicate that obesity is associated with increased bone mass due to excessive mechanical stress [1-9]. However, these data are controversial, and the risk of fractures in this group is higher [10-12]. Also, a significant number of people working in the industry are exposed to vibrations. Therefore, the number of patients with disorders of the musculoskeletal system grows. This leads to muscle hypertrophy, osteoarthritis, osteoporosis and calcination



of vertebrae, and the development of secondary radicular syndrome with trophic disorders of the extremities. Such changes mainly occur in two to five years of work with the constant exposure to vibration exceeding the maximum allowable levels. However, some literature sources point to the efficacy of moderate physical activity in increasing bone mass [13-15] despite such data. High-frequency whole-body mechanical oscillations can be used to prevent osteoporosis, increase bone mineral density, and reduce body fat percentage [15-22]. However, fracture risk factors depend on the mineral mass of its trabecular and cortical layers and its composite characteristics, i.e., bone micro-architectonics. Thus, to evaluate bone quality, we must consider the markers of bone remodeling, hormone panel, and bone micro-structure, which we determined an experiment using the x-ray diffraction method [23-24].

Our study aims to investigate the effect of high-frequency oscillations on the process of bone remodeling in rats with a sedentary lifestyle and obesity.

Materials and Methods

Animal model

The experimental study was performed on 54 Wistar male rats weighing 180-200 g, kept under the same vivarium conditions. All animal experiments were conducted in compliance with bioethical principles per the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Council Directive 86/609/EEC (1986), Law of Ukraine No. 3447-IV On the Protection of

Animals from Cruelty, General Ethical Principles of Animal Experiments, approved by the First National Congress of Ukraine on Bioethics (2001). The experimental rats were divided into three groups, 18 rats in each: the control group - standard vivarium conditions, the first experimental group - limited mobility condition + high-calorie diet (LMC+HCD), the second experimental group - LMC+HCD+WBV. The obesity condition was modeled through a high-calorie diet (C 11024, Research Diets, New Brunswick, NJ); the limited mobility condition was modeled using partition cages to restrict the rats' mobility. All experimental rats were weighed every two weeks. Vertical vibration oscillations were modeled using a 250 W vibrating table with the maximum pressure of 7 bar and 50 Hz frequency, $g = 0.3$ (Figure 1). After the 8th, the 16th and the 24th week, six animals from each group were removed from the experiment by decapitation under general intraperitoneal anesthesia at 0.3 g/kg.

Determining hormonal concentration

To assess bone metabolism in rat blood plasma, we determined the levels of parathyroid hormone and calcitonin using commercial EIA DRG-3645 PTH (Parathyroid hormone) Intact ELISA and EIA DRG-3648 Calcitonin ELISA (DRG International, Inc., USA). The study is based on the "sandwich" method (double-antibody method). Standards, controls, test samples and specific antibodies to the corresponding hormone labeled with biotin were added to the wells coated with PTH/CT antibodies. This forms the AB-PTH/CT-AB+Biotine complex. After the first incubation and washing phase, a chromogenic solution (tetramethylbenzidine) was added. The intensity of its color changes



Figure 1: Experimental design.

in direct proportion to the concentration of the sample's specific antibodies. We measured the color intensity at a wavelength of 450 nm.

X-ray diffraction

To study the ultrastructure of the tibia bone mineral component, we used the X-ray diffraction analysis method. The femur was dried at 110°C in a drying cabinet. The X-ray diffraction spectra of the samples were obtained on an automated X-ray diffractometer in Cu K α radiation ($\lambda=1.5418$ Å), monochromatized by reflection from a plane (002) of a single pyrographic crystal, mounted on a diffracted beam [23-27]. We used the Bragg-Brentano focusing scheme ($\theta - 2\theta$). The diffraction patterns were recorded in the continuous movement mode of the detector with an angular velocity of 20/min, a constant value of the integration time $\tau = 1$ s., x-ray tube voltage at $U = 26$ kV, and anode current at $I = 15$ mA.

Statistical analysis

Statistical analysis of the data was performed using StatSoft STATISTICA 8.0.360. In the STATISTICS package, the comparison of two average samples of normally distributed features (Student's t-criterion) was implemented in the Basic Statistics/Tables module. The t-test, independent, by variable submodule, was used for two different general summations.

Results

Rats' weight

The laboratory rats' weight in group I (HCD+LMC) increased to 194.63 ± 6.1 g in the 8th week, 309.45 ± 6.39 g in the 16th week and 340.82 ± 8.62 g in the 24th week, which indicates a statistically significant increase in weight while compared to the control group ($p = 0.014$). In the HCD+LMC+WBV group, the rats' weight increased to 198.3 ± 6.61 g, 279.0 ± 8.14 g, and 304.93 ± 5.07 g in the 8th, 16th, and 24th week, respectively ($p>0.05$), (Figure 2).

Leptin is the hormone involved in the regulation of body weight. While increasing weight by 10%, serum leptin levels may become more than 3 times higher. Therefore, to assess energy metabolism, it is advisable to determine its concentration. In the 8th week of the experiment, the leptin level in the control group was 5.25 ± 0.42 ng/ml; in experimental group I, it amounted to 15.01 ± 1.19 ng/ml ($p = 0.000007$), and in experimental group II – 11.13 ± 1.71 ng/ml ($p = 0.004$). In the 16th week of the experiment, the dynamics of leptin levels were the following: in the control group, it remained nearly unchanged at 5.91 ± 0.35 ng/ml, in experimental group I, the average level was at 21.01 ± 1.95 ng/ml ($p = 0.000008$), in experimental group II – 16.07 ± 1.84 ng/ml ($p = 0.00014$). In the 24th week of the experiment: the control group showed 4.93 ± 0.25 ng/ml, experimental group I – 24.51 ± 2.29 ng/ml ($p = 0.000003$), and experimental

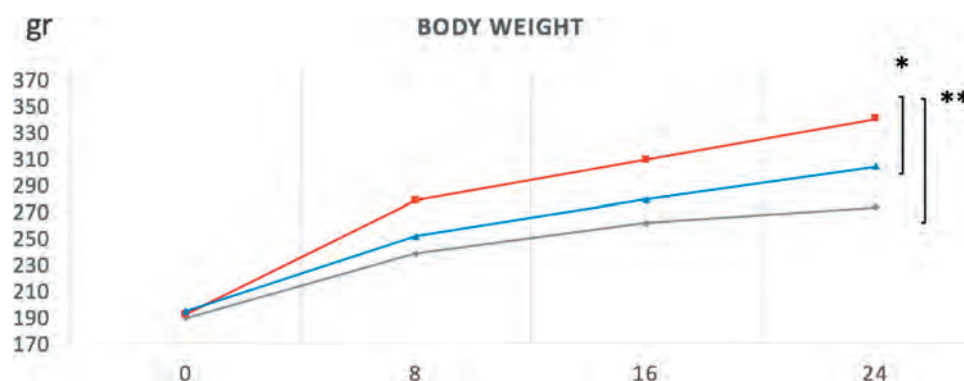


Figure 2: Body weight. * - groups do not differ statistically, $p > 0.05$; ** - groups differ statistically at $p = 0.014$. ■ - control group; ■ - limited mobility condition + high-calorie diet; ■ - limited mobility condition + high-calorie diet + WBV

group II - 18.07 ± 1.67 ng/ml ($p = 0.000007$) (Figure 3A).

For the evaluation of bone tissue remodeling of the experimental rats, we

examined hormonal markers of bone condition, particularly parathyroid hormone (PTH) and calcitonin. The blood analysis showed significant differences in PTH levels between

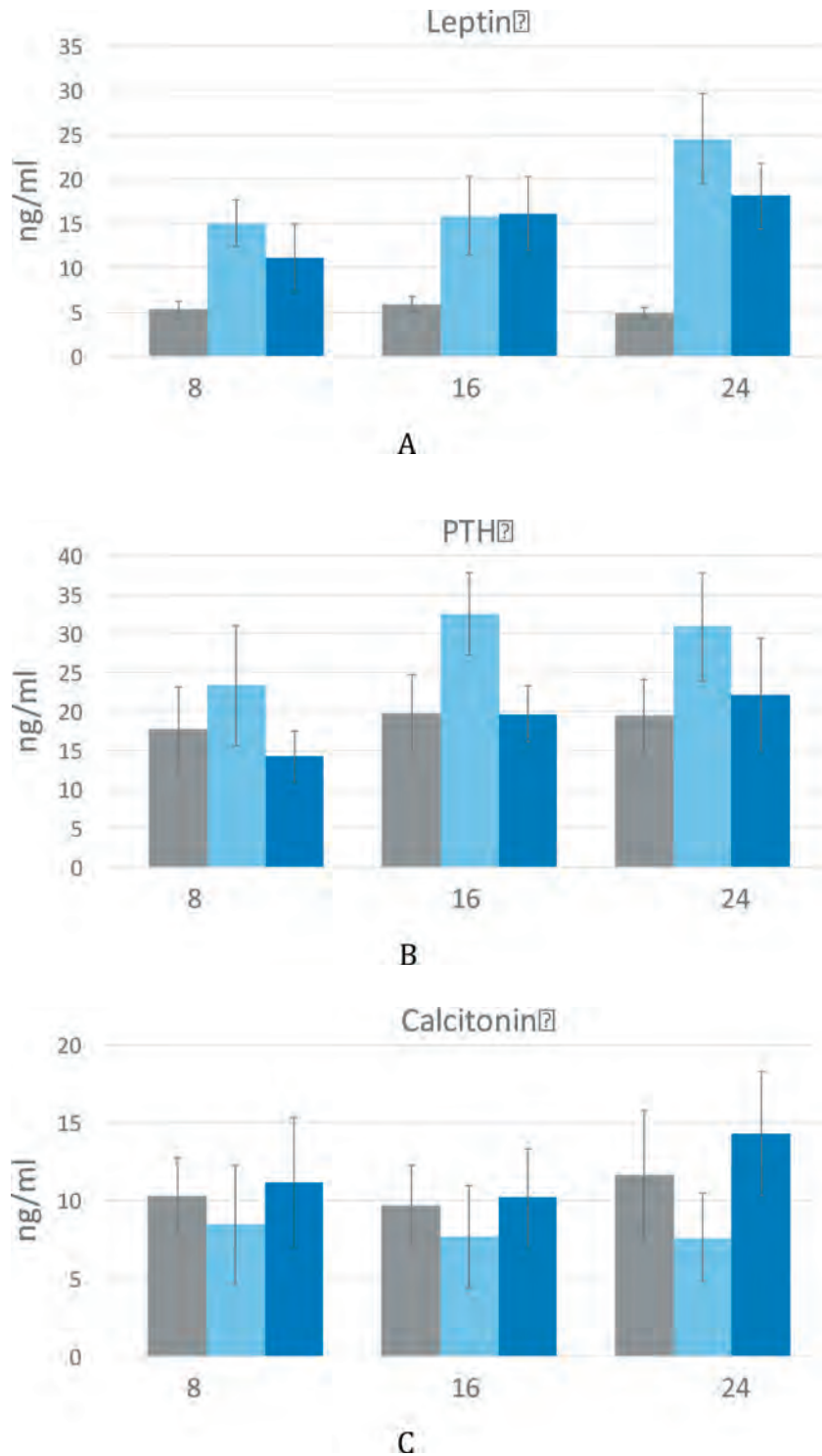


Figure 3: Cytokines levels and bone remodeling serum markers.

■ control group; ■ limited mobility condition + high-calorie diet; ■ limited mobility condition + high-calorie diet + WBV

(A - concentration of leptin, ng/ml; B - concentration of PTH, ng/ml; C - concentration of Calcitonin, ng/ml)

the control and experimental groups of rats (Figure 3B). In the 8th week of the experiment, the PHT concentration in the control group of animals was at 17.70 ± 2.42 pg/ml; in experimental group I, it was at 23.32 ± 3.45 pg/ml ($p=0.175$), and in experimental group II – 14.2 ± 1.5 pg/ml ($p>0.05$). In the 16th week of the experiment, there was the following dynamic of the parathyroid hormone level: in the control group, the figure remained almost unchanged and amounted to 19.72 ± 2.22 pg/ml; in experimental group I, it was at 32.5 ± 2.38 pg/ml ($p = 0.0015$), in experimental group II – 19.65 ± 1.62 pg/ml ($p>0.05$ for the control group and $p = 0.000633$ for the first experimental group). In the 24th week of the experiment, in the control group, the level was at 19.43 ± 2.05 pg/ml, in experimental group I – 30.92 ± 3.11 pg/ml ($p=0.007$), and in experimental group II at 22.08 ± 3.27 pg/ml ($p>0.05$).

In the 8th week of the experiment, the indicator of the rats' calcitonin level (Figure 3C) in the control group was at 10.3 ± 1.1 pg/ml, in experimental group I at 8.43 ± 1.7 pg/ml ($p>0.05$), in experimental group II – 11.18 ± 1.92 pg/ml ($p>0.05$). In the 6th week of the experiment, there were the following changes in the calcitonin level: in the control group, the figure remained practically unchanged at 9.67 ± 1.18 pg/ml, in experimental group I, the average level amounted to 7.68 ± 1.46 pg/mL ($p>0.05$), in experimental group II – 10.18 ± 1.4 pg/ml ($p>0.05$). In the 24th week of the experiment, in the control group, the figure was at 11.63 ± 1.86 pg/ml, in experimental group I – 7.62 ± 1.28 pg/ml ($p>0.05$), and in experimental group II – 14.3 ± 1.78 pg/ml ($p>0.05$).

Correlation analysis allowed us to establish the direct correlation ($p = 0.54$) between PTH and calcitonin in the 8th week of the experiment in the first group, the reverse correlation ($p = -0.77$) in the 16th week and the direct connection in the 24th week. In the 8th week of the experiment, the WBV groups showed a strong positive correlation ($p = 0.8857$), weak positive correlation $p = 0.25$ in the 16th week and direct positive correlation $p = 0.54$ in the 24th week of the experiment.

The diffraction patterns of the tibia series samples are shown in Figure 4 while compared to the theoretical diffraction pattern of the $\text{Ca}_{10}\text{P}_6\text{H}_2\text{O}_{26}$ chemical compound (hexagonal

syngony, space group P 63/m, unit cell parameters $a=9.42$ Å, $c=6.88$ Å). Significant erosion of the diffraction maxima of the $\text{Ca}_{10}\text{P}_6\text{H}_2\text{O}_{26}$ crystalline phase indicates a low degree of crystallinity of the compound due to the small size of the coherent scattering regions (the crystallite size does not exceed 10 nm). Also, a wide diffuse halo is observed in the diffraction patterns around the diffraction angle $2\theta \approx 21^\circ$, indicating the presence of an amorphous (disordered) phase represented by collagen fibers in the samples. In the series of samples, the highest content of the amorphous phase is observed in the samples of the first group in the 16th and 24th weeks of the experiment. The decrease in the intensity of the diffuse maximum of the samples obtained on the 8th day indicates a reduction of the amorphous phase contents. At the same time, on the 24th day, we can observe both the decrease of the diffuse maximum and the intensity of the crystalline phase maxima, which is particularly pronounced in the area of the most intense lines (211), (121), (112) and (300) of the $\text{Ca}_{10}\text{P}_6\text{H}_2\text{O}_{26}$ phase. To estimate the quantitative content of the amorphous and crystalline phases, we used the following ratios:

$$X_{am.} = \frac{I_{am}}{(I_{am} + I_{cr})}, X_{cr} = 1 - X_{am.}$$

(I_{am} – the maximum intensity from the amorphous phase, measured at $2\theta \approx 21.5^\circ$)

I_{cr} – the maximum intensity of the crystalline phase, measured at $2\theta \approx 32.1^\circ$, while taking into account the background scattering).

The calculations results are shown in Table 1. In the 8th wk. → 16th wk. → 24th wk. sequence, we can observe the increase of the crystalline phase volume fraction from 79% to 88/85% in the second experimental group and the decrease of the mineral component in the first experimental group (Figure 4 A/B/C).

Figure 4D displays a significant increase in the intensity of crystalline phase lines in diffraction patterns. Also, in the samples of the 16th day of the experiment, there is a noticeable decrease in the intensity of the diffuse background from the amorphous phase. According to the results of the phase content estimation,

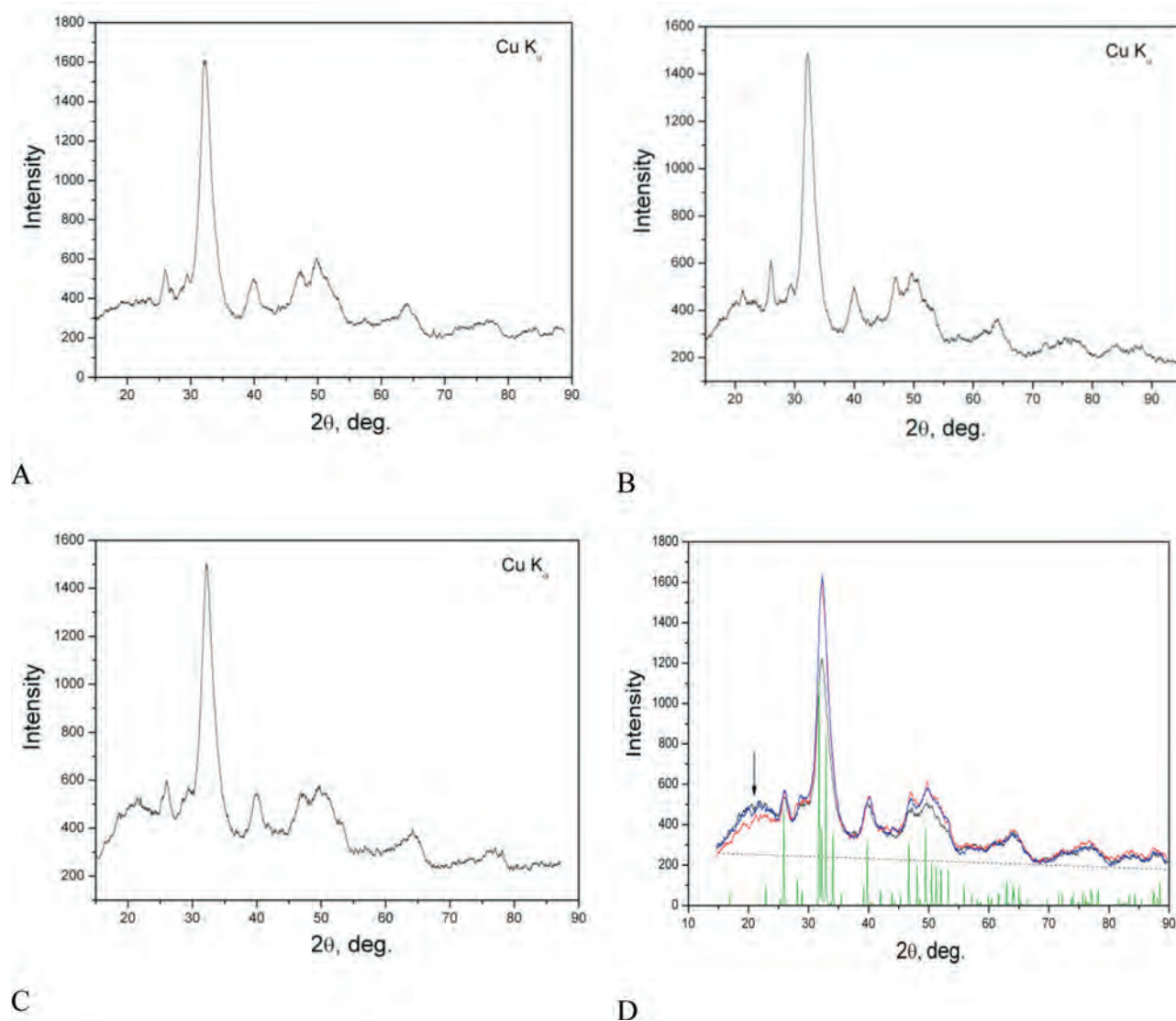


Figure 4: A, B, C: Diffraction patterns of the rat's femur in experimental group I (HCD+LMC) in the 8th, 16th and 24th week, respectively. To determine the integral intensity of the hydroxyapatite reflex, we chose the reflex of the 30-37° angular range since it is of the highest intensity. D – diffraction patterns of experimental group II samples (HCD+LMC+ WBV) in – 8th; – 16th ; – 24th week. The arrow indicates the position of the amorphous phase diffuse maximum, while the dashed line indicates the background scattering.

Table 1: Volume fractions of amorphous and crystalline phases of the samples.

Sample HCD+LMC	X_{am}	X_{cr}
8 th wk.	0.18	0.82
16 th wk.	0.2	0.80
24 th wk.	0.24	0.76
Sample HCD+LMC+WBV	X_{am}	X_{cr}
8 th wk.	0.21	0.79
16 th wk.	0.12	0.88
24 th wk.	0.15	0.85

the maximum increase in the crystalline phase content was observed in the WBV samples in the 16th and 24th weeks of the study.

Discussion

We used the presented model of obesity with a high-calorie diet to demonstrate that in the 8th week of the study, there already was a decrease of the mineral mass of trabecular and cortical layers of the rats' tibia, despite a significant increase in body weight of animals and increased mechanical stress. These structural changes

co-occur with an increasing level of leptin in serum and PTH. The mechanism of the mineral component loss is explained by the change in the correlation of RANKL/OPG expression levels of osteoblasts and the increased number of TRAP-positive osteoclasts. Such changes may be accompanied by increased activity of osteoclasts and reduced expression of anti-inflammatory cytokine IL-10. Obesity can enhance bone resorption through the regulation of proinflammatory cytokines, such as IL-6 and TNF- α , that stimulate the activity of the osteoclasts while regulating the RANKL/RANK/OPG path. This may further lead to chronic degenerative and inflammatory changes in the musculoskeletal system [28-31].

Obesity and a sedentary lifestyle might affect bone metabolism directly or indirectly through cytokines originating from adipocytes, particularly through leptin and adiponectin. This process is usually accompanied by a significant increase in serum leptin and a decrease in adiponectin [32]. Leptin's effect on the bone remains controversial. There are reports both on positive and negative effects. High concentrations of leptin in serum (as observed in our experimental animal model with obesity) may negatively affect bone metabolism. We have discovered that the increased level of leptin in serum is a negative regulator of bone mass [32, 33]. Moreover, of course, it should be noted that a high-fat diet, which often leads to obesity, prevents the absorption of calcium in the intestine. Free fatty acids can form calcium salt and contribute to low calcium absorption [29].

Nowadays, the WHO recommends moderate exercise as effective prevention of various diseases, including osteopenia. It decreases fat mass and increases bone mass (according to several experimental models with mice). High-frequency whole-body vibration is regarded as a means of mechanical influence that potentially improves the condition of the locomotor apparatus.

Besides cytokines, we conducted laboratory tests of the hormonal panel. Our research showed that in control (rats without obesity) and the HCD+LMC+WBV group, the mineral component amount was higher than in the HCD+LMC group. Besides, the first experimental

group showed a higher concentration of the parathyroid hormone in the serum (PTH) and lower calcitonin levels. The research results indicate that obesity has a negative impact on mineral bone mass, serum PTH, and calcitonin, partly explained in our conclusion. It is worth noting that the experimental animals showed decreased fat mass and the leptin concentration in blood serum.

We considered vertical whole-body vibration, as the means of mechanical stimulation, an alternative to pharmacological treatment of osteoporosis. Such stimulation induces the growth of the mineral component as a result of mechanical oscillations with micro-deformation of the bones. Previous studies presented by Rubin *et al.* have demonstrated an increase in the cortical and trabecular bone of turkeys subjected to vibration therapy [21, 22]. Some studies on animals and humans have proved positive effects of the vibration therapy on the structure and biomechanical properties of joints and muscles [14, 15].

Conclusion

Therefore, mechanical stimulation may serve as a promising alternative to medication therapy for osteopenia. While summarizing the data, our study provides a new understanding of the mechanisms of the mineral component loss in bone tissue in the case of obesity and sedentary lifestyle, while forecasting the future risks of osteoporotic fractures. The results of the experiment indicate that WBV is beneficial in preventing osteoporosis and bone mass loss.

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Ethical approval

Ethics Commission Report No. 10, 16.12.2019, Danylo Halytsky Lviv National Medical University.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

The Comparative Effect of Red Guava (*Psidium guajava. L.*) with Papaya (*Carica papaya*) on Blood Glucose Level of Type 2-Diabetic Patients

Zuhria Ismawanti^{1*}, JB. Suparyatmo², Budiyantri Wiboworini³

¹ Department of Nutrition, Faculty of Health Science Kusuma Husada University of Surakarta, Surakarta, Indonesia

² Department of Clinical Pathology, Faculty of Medicine Sebelas Maret University, Surakarta, Indonesia

³ Department of Nutrition, Faculty of Medicine Sebelas Maret University, Surakarta, Indonesia

*Correspondence to: Zuhria Ismawanti, Department of Nutrition, Faculty of Health Science Kusuma Husada University of Surakarta, Surakarta, Indonesia. E-mail: riazuhria27@gmail.com

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Abstract

Introduction: Diabetes mellitus (DM) is a metabolic disease with clinical manifestations of hyperglycemia. In DM, hyperglycemia increases free radical levels that lead to cell damage. Fruit antioxidants are used to prevent cell damaged due to their effect in suppressing lipid peroxidation and lipotoxicity. This study aimed to evaluate the effect of red guava and papaya antioxidants in reducing blood glucose levels in type 2 DM patients **Material and Methods:** This was a quasi-experimental study with a pre-test post-test control group design. Thirty-nine subjects were divided randomly into 3 groups: red guava group (282.2 gr), papaya group (302.4 gr) and control group (only given mineral water). The treatment was conducted for 14 days. The data were analyzed using the One-way ANOVA test, continued with post hoc tests. **Results:** There was a significant decrease in blood glucose level after the administration of red guava (33 g/dl) and papaya (14 g/ dl) compared to the control group ($p < 0.05$), but there were no significant differences ($p > 0.05$) between red guava and papaya. **Conclusions:** It can be concluded that red guava and papaya are equally effective in reducing blood glucose levels in type 2 DM patients.

Keywords: Red guava, papaya, antioxidant, blood glucose.

Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by hyperglycemia caused by impaired insulin secretion, insulin action, or both. DM causes various chronic complications of the eyes, kidneys, nerves, and blood vessels [1-2]. After India (32.7 million), China (22.6 million) and the United States (15.3 million), Indonesia has the highest number of people with diabetes (14 million). Riskesdas shows that East Java, one of the provinces in Indonesia, has a DM prevalence of 2.5% [3]. Based on data obtained from the Jombang District Health Office, the prevalence of diabetes has increased for three consecutive

years, namely in 2015 by 5.0%, in 2016 by 5.71%, and in 2017 by 6.21% [3-6].

In diabetic patients, insulin cannot work effectively so that glucose from the consumed food cannot be maximally converted into energy and glycogen, leading to elevated blood glucose levels [7].

The autoxidation process of hyperglycemia triggers the formation of free radicals. Free radicals can damage the cell membrane into lipid peroxide and cause oxidative damage. Antioxidants intake is intended to suppress lipid peroxidation. Therefore lipotoxicity can be prevented, and cell damage caused by hyperglycemia can be avoided [8]. Vitamin C is one of the antioxidants



that modulates insulin action in DM patients and is associated with a decrease in blood glucose levels [9].

Red guava (*Psidium Guajava* Linn.) and papaya (*Carica Papaya*) are fruits that contain various nutritional contents, including vitamin C, fiber, carbohydrates, and flavonoids [10, 11]. Guava has 87 mg of vitamin C and papaya has 78 mg per 100 grams of fruit. Vitamin C decreases insulin resistance by improving endothelial function and reducing oxidative stress [12].

The ultimate nutritional content of guava shows that it is rich in flavonoids, particularly quercetin. [13]. Quercetin can improve glucose uptake by stimulating 3T3-L1 in mature adipocytes by insulin [14]. Moreover, it can escalate insulin sensitivity by increasing tyrosine phosphorylation in the insulin receptor and extending the signaling process. Both of these mechanisms indicate that quercetin can improve insulin resistance in peripheral tissues [15].

Papaya contains saponins with hypoglycemic effects through a mechanism for increasing glucose uptake by cells, insulin release, and insulin sensitizer [16, 17]. Guava and papaya have a glycemic index of 19 and 25, respectively, which can be categorized as a low glycemic index. The low glycemic index can reduce insulin resistance and insulin levels, causing a decrease in free fatty acids that prevent the accumulation of blood triglycerides [18].

Besides being rich in nutrients, red guava and papaya are cheap, ubiquitous throughout the year. This study aimed to examine the effect of papaya and red guava in reducing blood glucose levels.

Material and Methods

Study design and patients

This was a quasi-experimental study with a pre-test post-test control design. The independent variable in this study was the administration of red guava and papaya, and the dependent variable was blood glucose levels. The study population included type 2 DM patients of the Jombang district health center. The subjects were selected

by a simple random sampling method, which met the inclusion criteria: age between 45-70 years; HbA1c value greater than 7; possibility to chew fruits; smoke and alcohol-free. Subjects who used insulin or suffered from gastrointestinal/liver/ heart diseases were excluded. The sample size calculation was done using the hypothesis test formula on the average of two independent samples.

Intervention

Thirty-nine subjects were divided into three groups: 13 subjects were treated with 282.2 grams of red guavas, 13 subjects were given 302.4 grams of papaya and 13 were treated with mineral water as a control group. The experiment was conducted for 14 days.

The fruits were consumed as a snack after breakfast and lunch. The subject's compliance in consuming the fruits was observed using a compliance form. All subjects were given counseling and leaflets about DM at the beginning of the study. Age and sex data were obtained using a questionnaire. Body mass index (BMI) was calculated using Weight/Height in kg/m². Food intake was collected two times during the intervention, using 24-hour food recall forms and analyzed using the Nutrisurvey software to get average energy, protein, fat and carbohydrates intake. Blood samples were taken twice, before and after the intervention by laboratory staff after subjects fasted for $\pm$ 8 hours. Fasting blood glucose levels were measured using the hexokinase method and categorized as good if the levels were between 80 and 100 mg/dl, moderate if the values were 100-125 mg/dl, and bad if the value was higher than 126 mg/dl.

Statistical analysis

The normality of data was tested using Shapiro Wilk. Differences in fasting blood glucose levels before and after intervention in each group were tested using the paired t-test. The One-way ANOVA test was used to determine the differences in blood glucose level reduction among groups and continued with the post hoc

test to analyze which treatment was significant. This research has been approved by the Ethics Committee of the Sebelas Maret University FK No. 305/UN27.6/KEPK/2018.

Results

Only 38 subjects could be analyzed because one subject from the red guava group dropped out. The subjects were female and male, aged 45-70 years. Table 1 shows that the average energy, protein, fat and carbohydrate intake of each group had considerable variation. However, there were no significant differences between the red guava, papaya, and control groups ($p > 0.05$).

Table 2 shows that the most significant reduction in fasting blood glucose levels occurred in the red guava group. The value of $p = 0.001$ ($p < 0.05$) meant that there was a significant difference in decreasing fasting blood glucose levels among the three treatment groups.

The post hoc test showed that there were significant differences in fasting blood glucose levels between the red guava intervention group compared to control ($p = 0.000$), and between the papaya intervention group and control ($p = 0.000$). However, the decrease in fasting blood glucose levels between red guava and papaya was not significantly different ($p = 0.140$). This meant that red guava and papaya were equally effective in reducing fasting blood glucose levels.

Discussion

The administration of red guava and papaya for 14 days could reduce fasting blood glucose levels in DM patients. Huang et al. showed that red guava significantly reduced hyperglycemia in STZ-induced diabetic rats [19]. Red guava could maintain insulin secretion capacity and the ability of pancreatic β cells. Antioxidant activity of red guava might have an antihyperglycemic

Table 1: Average nutrients intake.

	Intake	Red Guava Mean $\pm$ SD	Papaya Mean $\pm$ SD	Control Mean $\pm$ SD	p
Female	Energy (Kcal)	1853 $\pm$ 330	1966 $\pm$ 376	2003 $\pm$ 346	0.660
	Protein (gram)	44 $\pm$ 14	58 $\pm$ 24	52 $\pm$ 17	0.477
	Fat (gram)	100 $\pm$ 38	118 $\pm$ 31	87 $\pm$ 23	0.09
	Carbohydrates (gram)	210 $\pm$ 70	220 $\pm$ 72	228 $\pm$ 67	0.604
Male	Energy (Kcal)	2164 $\pm$ 228	2053 $\pm$ 45	2108 $\pm$ 172	0.460
	Protein (gram)	60 $\pm$ 20	56 $\pm$ 28	55 $\pm$ 19	0.966
	Fat (gram)	161 $\pm$ 21	145 $\pm$ 31	137 $\pm$ 19	0.291
	Carbohydrates (gram)	235 $\pm$ 99	220 $\pm$ 58	220 $\pm$ 89	0.977

Note: The Value of One-way ANOVA tests show the difference in the average intake of the 3 groups.

Table 2: Differences in Fasting Blood Glucose Levels (mg/dl).

Groups	Pre Mean $\pm$ SD	Post Mean $\pm$ SD	Δ Pre-post Mean $\pm$ SD	p1	Post hoc	p2
Red guava	206 $\pm$ 41	172 $\pm$ 41	33 $\pm$ 2	0.001	Red guava vs. Control	0.000
Papaya	200 $\pm$ 33	186 $\pm$ 33	14 $\pm$ 0.9		Papaya vs. Control	0.000
Control	187 $\pm$ 38	181 $\pm$ 33	6.8 $\pm$ 5		Red guava vs. papaya	0.140

Note: p1- p value of One-way ANOVA tests; p2 - p value of post hoc tests.

effect in STZ-induced diabetic rats. Sureddi showed that papaya significantly reduced plasma glucose levels in STZ-induced alloxan diabetic rats [20]. Papaya could increase insulin secretion from pancreatic β cells and glucose absorption by tissues. Post hoc analysis showed a significant difference in decreasing fasting blood glucose levels between the red guava and control groups ($p = 0.000$), and between papaya and control ($p = 0.000$). However, fasting blood glucose levels between red guava and papaya were not different significantly ($p = 0.140$). It means that red guava and papaya were similarly effective in reducing fasting blood glucose levels, but red guava had a higher clinical effect in reducing fasting blood glucose levels. After the administration of red guava, the decrease in blood glucose level is 2.3 times greater than in papaya (33 ± 2 compared to 14 ± 0.9 g/dl, respectively).

Red guava and papaya contain vitamin C or ascorbic acid, which can affect blood glucose levels [21-24]. Vitamin C is an antioxidant, which plays an essential role in protecting against damage from free radicals. Vitamin C is structurally similar to glucose and can interchange it in many chemical reactions and effectively prevent the glycosylation of non-enzymatic proteins. Antioxidants increase β cell function in diabetes, although the effect may not be through a direct action on the β cells. Increased action of vitamin C on insulin may be due to an increase in non-oxidative glucose metabolism [12].

Red guava contains fiber and quercetin. Quercetin can reduce blood glucose levels due to its potential effect as an inhibitor of glucose transport by intestinal GLUT2 and GLUT5. These receptors are responsible for glucose absorption in the small intestine [24]. Fiber consumption has a positive effect on blood glucose levels in type 2.

DM patients. Dietary fiber delays gastric emptying time and intestinal glucose absorption. Studies on type 2 DM patients from Texas reported that a high-fiber diet would reduce blood glucose levels [21, 22].

Another beneficial content of papaya in diabetes is represented by saponins [25]. Saponins are antidiabetics through their effect of inhibiting the α -glucosidase enzyme, an enzyme that plays a role in converting carbohydrates

to glucose. If the α -glucosidase enzyme is inhibited, the glucose (sugar) levels in the blood will decrease, resulting in a hypoglycemic effect (blood sugar levels decrease) [26]. Saponins are known to have hypoglycemic effects indicated by increasing glucose uptake by cells, insulin release, and effects (insulin sensitizer) [16-17]. Saponins stimulate insulin signals in diabetic mice, resulting in improved homeostasis [27]. A limitation of this study was that we did not analyze other nutrients intake that can decrease blood glucose levels.

Conclusion

Giving 282.2 grams of red guava and 302.4 grams of papaya for 14 days were equally effective in reducing blood glucose levels of type 2 diabetes mellitus patients.

Conflict of Interest

The author declares no conflict of interest.

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Original Research

Clinical Case of Peripheral Artery Disease with Post-Thrombotic Disease in a Patient with Type 2 Diabetes Mellitus

Svetlana Mykolayivna Gramatiuk¹, Irina Yuriivna Bagmut², Julia Viktorivna Ivanova³,
Michael Ivanovich Sheremet⁴, Oleg Aleksandrovich Zarudny⁵, Kirill Valeryevich Miasoiedov³

¹ Institute of Cellular Biorehabilitation, Kharkiv, Ukraine

² Department of Clinical Pathophysiology, Topographic Anatomy and Operative Surgery, Kharkiv Medical Academy of Postgraduate Education, Kharkiv, Ukraine

³ Institute of General and Urgent Surgery of National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine

⁴ Surgery Department No. 1 of Bukovinian State Medical University, Chernivtsi, Ukraine

⁵ State Institution Zaytsev V.T. Institute of General and Urgent Surgery of National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine

*Correspondence to: Svetlana Mykolaivna Gramatiuk, Institute of Cellular Biorehabilitation, Kharkiv, Ukraine.

E-mail: gramatyuk@ukrainebiobank.com. Phone: +38(099)1549144

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Abstract

Introduction: Mesenchymal stem cells have the capacity of multidirectional differentiation and reduced immunogenicity; these cells are widely used in the field of regenerative medicine research. Our aim was to study the effect of stem cell transplantation on the course of the wound process in conditions of combined lesions of the arteries and veins of the lower extremities in a patient with type 2 diabetes mellitus. **Material and Methods:** The study presents Patient S., burned in 1952, with a 20-year history of type 2 diabetes. In our protocol, we used autologous mesenchymal stem cells isolated from the patient's peripheral blood by magnetic separation using an AutoMACS automatic system (USA). **Results:** The clinical case shows that in patients with combined lesions of the arteries and deep veins and large trophic ulcers, it is advisable to use a step-by-step treatment: first step - the restoration of the arterial blood flow in the extremity; second step - scleroobliteration of the incompetent perforator veins and the third step - transplantation of mesenchymal stem cells into muscles along the perimeter of trophic ulcer. **Conclusions:** This tactic contributes to the long-lasting and sustained positive clinical effect and rapid healing of trophic ulcers in patients with type 2 diabetes.

Keywords: Autologous mesenchymal stem cells, large trophic ulcers, patient with type 2 diabetes.

Introduction

According to various authors, obliterating atherosclerosis of the lower extremities' arteries is clinically manifested in 2–3% of the total population, and about 15–20% of the elderly (average age is 68 ± 5.5 years). In fact, the number of such patients is 3–4 times greater due to subclinical forms [1, 2, 10–13]. In 60–80% of patients with multiple and distal forms of pathology, severe ischemia

develops, leading to limb amputations in 10–20% cases. In the first three years after high amputations, mortality rates increase annually to 69.5% in patients with chronic critical ischemia of the lower extremities [3, 4–6, 14, 18, 22].

Due to increased number of patients with atherosclerotic lesions of several arterial beds in which arterial reconstructive surgery of the lower extremities is impossible or at high risk, as well as a large percentage of patients



with multilevel or distal lesions of the arteries of the lower extremities, the revascularization of lower extremities is not possible in all cases. The absence of an adequate microcirculatory bed is one of the causes of early postoperative thrombosis of bypass shunts [1, 7–9, 11, 23–25].

Therefore, the problem of treating patients with distal lesions of arteries of the lower extremity is one of the most challenging in vascular surgery. Its solution will not only save the limb and improve the quality of life for inoperable patients but also significantly extend their life span [10, 15–17, 20, 26].

Extremely unfavorable are the situations of combined lesions of the limb arteries with chronic venous insufficiency. Venous hypertension is one of the factors in the development of trophic disorders and initiates a whole cascade of pathological responses at subcellular, cellular, and tissue levels. According to the literature, trophic venous ulcers account for more than 70% of all lower extremity ulcers and are found in every fifth patient with chronic venous insufficiency. Using standard conservative therapy that lasts up to 4 months, healing of venous trophic ulcers occurs only in 50% of cases, 20% does not heal after two years of treatment, and after five years in 8% of patient's ulcerative defect remains open [5, 7, 15, 19, 27–29]. The combination of chronic venous insufficiency with chronic arterial disease significantly reduces the effectiveness of traditional treatment of venous trophic ulcers.

The search for new methods of complex treatment of trophic ulcers of the lower extremities is a topical issue of modern medicine, because despite the constant work of morphologists, surgeons and pharmacists to predict the development of the wound process, its healing remains almost an unattainable goal [8, 9, 17, 30].

Nowadays, the methods of stem cell transplantation of various origins in limb ischemia, pancreatic necrosis, diabetes mellitus, tumor hematology, and other diseases are being used in medicine more frequently [7–9, 21, 22]. A certain similarity was observed in the sequence of expression of marker genes and proteins after studying the directed differentiation of stem cells in vitro and in vivo. These cells have the ability to differentiate into different cell types and repair

tissues of damaged organs: liver, heart, skin, blood vessels, bones, and cl. [8, 11, 19]. Recent studies have shown that mesenchymal stem cells have the capacity for multidirectional differentiation and poor immunogenicity; these cells are widely used in regenerative medicine research.

However, there is little data in the literature regarding the scientific search for the possibility of using cellular technologies to treat patients with trophic ulcers.

The purpose of the study is to investigate the effect of stem cell transplantation on the course of the wound process in conditions of combined lesions of the arteries and veins of the lower extremities in a patient with diabetes.

In our protocol, we used autologous mesenchymal stem cells (MSC) isolated from the patient's peripheral blood by magnetic separation using an AutoMACS automatic system (USA). MSCs met the following criteria for expression of cell surface markers: $\geq 95\%$ of the population should express CD105, CD73, and CD90, and $\leq 2\%$ did not express CD45, CD34, CD14 or CD11b, CD79a or CD19, and MSC class II HLA were able to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [23, 24].

Case Presentation

Patient S. was admitted to the Department of acute vascular diseases of State Institution "V. T. Zaitsev Institute of General and Urgent Surgery of National Academy of Medical Sciences of Ukraine", complaining of a trophic ulcer on the left ankle (Figure 1), pain in both lower extremities (more exacerbated in the right foot and lower leg), which increase significantly during exercise, and feeling of cold in the fingers. Patient height and bodyweight were 162 cm and 71 kg (body mass index = 26.3), respectively. He was burned in 1952 and had a 20-year history of type 2 diabetes. The patient noted a worsened sleep at night, which was associated with unpleasant sensations and pain in both lower extremities. The length of painless walking distance was about 35–50 m, after which severe pain appeared in the calf muscles of both lower extremities (greater to the right).



Figure 1: Patient S. Trophic ulcer of the right calf.

History of the disease

The patient first felt the pain in the lower extremities during exercise three years ago. She was treated conservatively at a regional hospital. Two years ago, she had acute deep vein thrombosis of the lower extremities. The trophic ulcer appeared nine months prior to being admitted to the hospital. Traditional conservative and topical treatment was unsuccessful as the wound area increased.

During the physical examination, it was noted that the skin was pale and cold on both the lower legs and feet with moderate swelling of the shin. Active and passive movements of the right

and left joint extremities were preserved. While the femoral pulse was preserved in the right leg, pulsation of the popliteal, posterior tibial and anterior tibial arteries was absent. Transcutaneous oxygen pressure (TcpO₂) of the foot was 9.4 mmHg. Angiography and revascularization of the right lower extremity was performed on 05/10/2019 (Figure 2).

After revascularization, TcpO₂ became 39.6 mmHg. Scleroobliteration of incompetent perforator veins under ultrasound control was performed on 10/08/2019 (Figure 3). Aethoxysklerol 0.1–3% was used as a sclerotherapy agent and the sclerosing foam was obtained by the Tessari method (air- sclerosant ratio of 4:1).

Considering the size of the trophic ulcer, it was decided to include in the treatment regimen autologous mesenchymal stem cells which were isolated from the patient's peripheral blood using a magnetic separation method and cultured according to the standard protocol using a CO₂ incubator of 5% atmosphere with a 37°C temperature, with further trypsinization and technologic subculturing of cells cultures. The isolation, cultivation, and preservation of mesenchymal stem cells was performed on the basis of the Institute of Cellular Biorehabilitation, licensed by the Ministry of Health of Ukraine for the Bank of Cord Blood, Cells and Tissues (Order of the Ministry

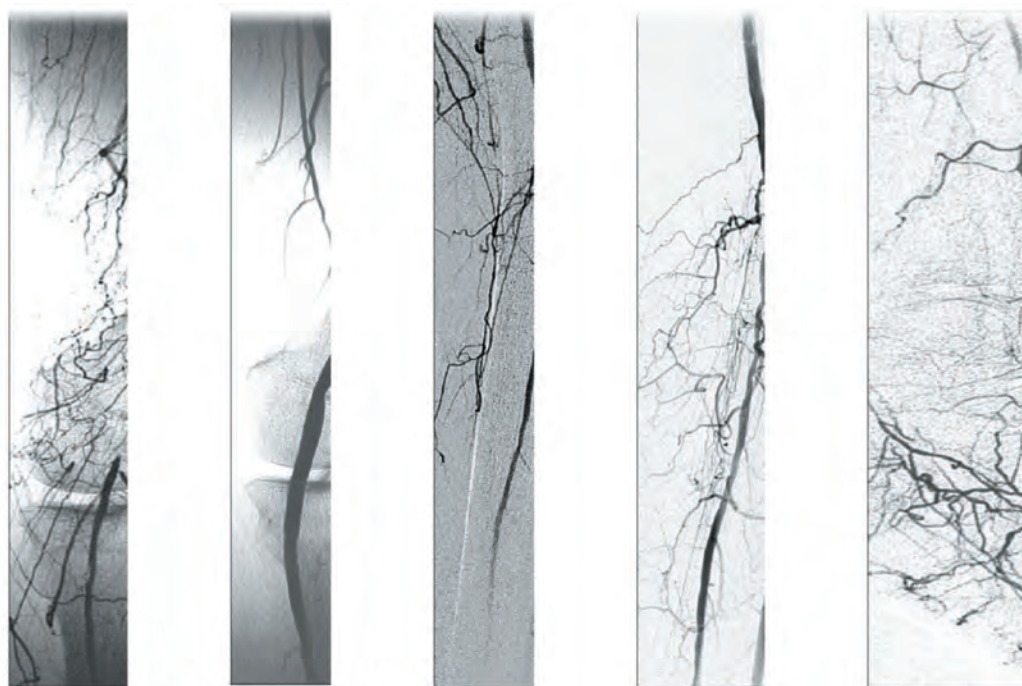


Figure 2: Stenting of the superficial femoral and popliteal artery, balloon angioplasty of the anterior tibial artery.



Figure 3: Scleroobliteration of incompetent perforator veins (ultrasound).

of Health of Ukraine No. 1813 dated 04/10/2018 and order of the Ministry of Health of Ukraine on 18.04.2019 No. 877) and is accredited according to the international standard ISO 20387: 2018 - biobank and biotechnology.

During standard therapy (surgical debridement: end result of non keratotic wound edges with a well-vascularized tissue bed; after the debridement was successful, local antimicrobial therapy was sufficient to eliminate bacteria in the ulcer), post-usual therapy local injections of autologous mesenchymal stem cells were used for four days.

On 10/10/2019, transplantation of stem cells into the leg's muscle tissue along the border of the trophic ulcer was performed (Figure 4). The dose was 5 ml of suspension containing 5.0×10^5 cells per 1 cm² of wound area. The pre-cell suspension was tested for the absence of pathogens. Negative results of serological and viral tests - HIV-1 and 2 (HIV Ag/Ab); Hepatitis B Virus Infection, Anti-hepatitis B core antigen (Anti-HBc);

Hepatitis C Virus Infection, Anti-HCV; Syphilis specific tests were obtained.

Cell therapy was performed according to the following protocol: after division, the characteristics and subculturing of the stem cells were introduced four times into the ulcer zone in eight points.

The patient was discharged from the hospital with recommendations for local treatment of the wound with ointments based on fat (methyl-uracil 10% ointment), the use of dual disaggregate therapy, blood pressure and glycemic control. Patient monitoring was performed every two weeks for up to 12 weeks of outpatient follow-up.

One month after the cell transplantation, the epithelization of approximately one-third of the wound area was noted (Figure 5).

Two months post cell transplantation, the patient's local status and general condition were improved, had increased ability for working and general activities (Figure 6). Also, TcpO₂ was 42.3 mmHg.



Figure 4: Stem cell transplantation in the muscle tissue of the calf.



Figure 5: One month after stem cell transplantation.



Figure 6: Two months after stem cell transplantation.

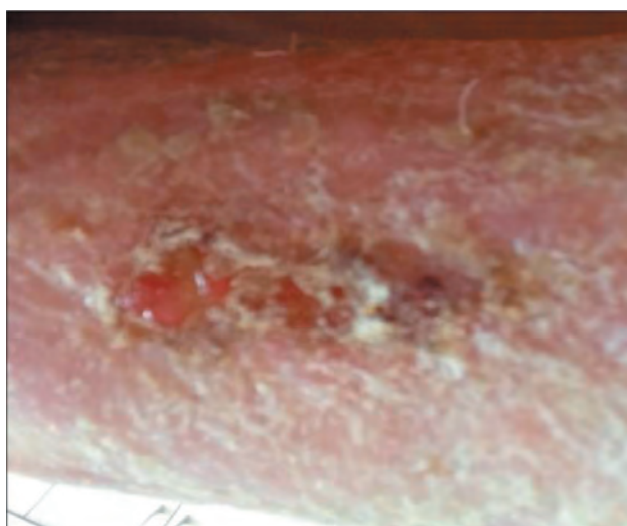


Figure 7: Four months after stem cell transplantation.

The first signs of wound healing were observed four months after the start of therapy (Figure 7). After cell transplantation, the condition of the patient was good, and working ability was preserved, allowing an active life. There was no limb edema, and the wound healed completely.

Discussion

Thus, the above clinical case shows that in patients with combined lesions of the arteries, deep veins and the presence of large trophic ulcers, it is advisable to use a step-by-step treatment: first step - restoration of the arterial blood flow in the affected extremity, second step - scleroobliteration of the incompetent perforator veins (Figure 3) and the third step - transplantation of

mesenchymal stem cells into the muscles along the perimeter of the trophic ulcer (Figure 4). This tactic contributes to the long-lasting and sustained positive clinical effect and rapid healing of trophic ulcers in patients with diabetes type 2.

Conclusion

In the initial stages of diagnosis, the multidisciplinary team should make treatment recommendations based on the severity of ischemia and the presence of infectious lesions. Multidisciplinary collaboration and optimization of treatment regimens are necessary if there is a long-term possibility of preserving any part of the foot or leg. It is clinically proven that the use of mesenchymal stem cell transplantation for patients with combined lesions of the lower extremity arteries and veins significantly increases the chances of successful treatment of trophic ulcers and chronic wounds. It is promising to study the effectiveness of the proposed method on a larger contingent of patients.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Sirtuin 1, but not Osteocalcin, Correlates with Lipid Accumulation Product, Visceral Adiposity and Atherogenicity Indices in Newly Diagnosed Prediabetes-Metabolic Syndrome Patients

Violet Kasabri*, Abba AlBsoul-Younes, Omar Rizik, Maysa Suyagh, Kashma Omed, Govand Yassin, Sundus AlAlawi

School of Pharmacy, University of Jordan, Amman, Jordan

*Correspondence to: Violet Kasabri, School of Pharmacy, University of Jordan, Queen Rania Street, Amman 11942, Jordan.
E-mail: hotice162@gmail.com. Phone: +96265355000. Fax: +96265300250

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Abstract

Introduction: Osteocalcin (OCN) and Sirtuin 1 (SIRT1) are intricately involved in metabolic syndrome (MetS) and prediabetes (PreDM) anomalies and derangements. In a heterogeneous pool of nondiabetic and preDM MetS recruits, adiposity, atherogenicity and blood indices, SIRT1 and OCN were compared to the respective parameters in normoglycemic and lean controls. Further testing of putative relationships between indices and markers was performed in 59 patients with MetS. **Material and Methods:** In this cross-sectional study, comparisons and correlations were undertaken for biomarkers, adiposity, atherogenicity and hematological indices in 29 MetS-normoglycemic and 30 newly diagnosed drug-naïve MetS-preDM patients versus 29 lean, healthy and normoglycemic controls. ANOVA and Spearman rank correlations were used for statistical comparisons. **Results:** OCN level (OCN; ng/mL) was significantly higher in normoglycemic MetS vs. both MetS-PreDM and controls (28.13 ± 1.22 and 26.02 ± 3.2 vs. 23.3 ± 3.19 , $P < 0.001$ respectively). In contrast, the circulating level of SIRT1 (ng/mL) was lower in both normoglycemic and preDM MetS vs. healthy controls (1.42 ± 0.47 and 1.64 ± 0.58 vs. 3.88 ± 0.95 ; $P < 0.001$, respectively). Except for fasting plasma glucose and glycated hemoglobin (A1C), no further intergroup discrepancy could be identified between normoglycemic-MetS and preDM-MetS. Notably, adiposity indices and the atherogenicity index of plasma were significantly higher in both MetS (normoglycemic and preDM) groups vs. controls. The LDL-C/HDL-C ratio, visceral adiposity index, and waist/hip ratio were higher only in MetS-preDM vs. controls. In the MetS pool ($n=59$), OCT, but not SIRT1, was associated reciprocally with fasting glycemia and A1C, monocyte/lymphocyte ratio, but proportionally with HC. In the same MetS pool, SIRT1 correlated significantly positively with TG, lipid accumulation product, visceral adiposity index and the atherogenicity index of plasma. **Conclusions:** OCN and SIRT1 may reciprocally participate in the development of MetS and preDM; both biomarkers may be putatively surrogate diagnostic/prognostic tools for metabolic anomalies prediction/prevention and pharmacotherapy.

Keywords: Sirtuin 1, osteocalcin, prediabetes and metabolic syndrome, adiposity, hematology and atherogenicity indices.

Introduction

Diabetes mellitus (DM) is a constellation of progressive metabolic and endocrine derangements and anomalies [1]. Individuals with prediabetes (PreDM) have a glycosylated hemoglobin (HbA1C) value of 5.7–6.4%, impaired fasting glucose (IFG) and/or impaired glucose tolerance

(IGT). PreDM, as a risk factor for cardiovascular disease (CVD) and diabetes, correlates with abdominal obesity, hypertension (HTN), atherogenic dyslipidemia, and visceral obesity [1]. As stated by the International Diabetes Federation (IDF) [2], MetS is principally identified by central obesity (measured by waist circumference (WC) in relevance to gender and ethnicity) with two



or more anomalies of MetS components. MetS is heterogeneous due to its components' diversity, including dropped high-density lipoprotein cholesterol (HDL-C), raised triglyceride, raised blood pressure (BP) and/or raised fast blood glucose (FBG) levels [3].

Sirtuin 1 (SIRT1) belongs to the NAD⁺-dependent histone deacetylases (HDAC) family that prevents DNA transcription. In general, sirtuins predominantly regulate metabolism through their regulation of inflammation, oxidative stress and mitochondrial function, thus improving insulin resistance and T2DM. SIRT1 levels have an inverse relationship with cardiac performance and inflammatory cytokines in subcutaneous abdominal fat in overweight prediabetic patients [4]. SIRT1 can also be involved in the diversity of neurodegeneration, tumors, cardiomyopathies, arrhythmias, hypertension, dysregulation of insulin secretion from the pancreatic β -cells, endothelial dysfunction, atherosclerosis, MetS, obesity with fatty liver, DM, and dyslipidemia [5].

As a serum marker of bone formation, osteocalcin (OCN) is produced by osteoblasts. Low serum osteocalcin concentration is associated with lower bone remodeling and/or decreased bone quality in T2DM women with osteoporosis. The osteopontin-osteocalcin (OCN)-osteoprotegerin hormone triad involved in bone remodeling may affect glucose metabolism in preDM before overt T2DM occurs. Serum OCN concentration is negatively correlated with FPG, insulin resistance, and HbA1C. Furthermore, MetS is related to abdominal obesity, dyslipidemia, hyperglycemia, and hypertension and is substantially associated with osteoporosis. Contradictory reports delineated the lack of interaction among the skeletal bone-derived factors (OCN, Fibroblast growth factor 23, neutrophil gelatinase-associated lipocalin, Lipocalin-2) with visceral obesity and body mass index (BMI) [6–7]. This study aimed to compare and correlate Osteocalcin (OCN) and Sirtuin 1 (SIRT1) plasma levels, a set of clinical parameters, MetS related-adiposity, atherogenicity and hematological indices between normoglycemic MetS and newly diagnosed drug-naive prediabetic MetS patients vs. lean, apparently healthy and normoglycemic controls.

Material and Methods

Study design

This cross-sectional study was conducted to examine the comparison and relation between plasma levels of OCN and SIRT1 in three groups of the Jordanian population:

1. Control group: 29 participants were apparently healthy and lean (BMI < 25 kg/m²), and did not have hyperglycemia. A1C < 5.7%, and FPG < 100 mg/dL [1] were mainly considered for comparison purposes;
2. MetS-normoglycemic group: 29 participants; patients with central obesity plus ≥ 2 MetS components [1, 2] were either overweight (BMI > 25 kg/m²) or obese (BMI > 30 kg/m²). A1C < 5.7%, FPG < 100 mg/dL;
3. MetS-PreDM group: 30 participants; prediabetic patients were overweight or obese with central obesity plus ≥ 2 MetS components [2], but necessarily defined as drug-naive subjects (Figure 1).

Individuals with any of the following criteria were excluded from the study:

- Fasting for less than 12 hours.
- Females who are breastfeeding or pregnant.
- Any previous treatment with antidiabetic, anti-hyperlipidemic, or antihypertensive agents.
- Clinical evidence of autoimmune or life-threatening disease (drug abuse/alcohol/recently diagnosed with an untreated endocrine disorder).
- Patients with autoimmune or chronic inflammatory diseases.
- Obesity secondary to endocrine disorders, other than DM.

Study sample size

Sample size had been calculated by the following formula [8]:

$$N = \frac{2 \times SD^2 \times (z_{\alpha} + z_{\beta})^2}{\Delta^2}$$

Where:

N: Sample size

Z_{α} : Type one error= 1.96 when $\alpha = 5\%$

Z_{β} : Type two error= 1.28 when $\beta = 10\%$

SD = Standard deviation of OCN from Magalhães et al. [9a] study; equals 4.60 ng/mL

Δ = the difference between OCN levels in the MetS group vs. the control group in this observational study equals to 3.91 ng/mL. Thus, the minimum required number of participants per each group was 29 for OCN (Figure 1).

Clinical setting and metabolism-related indices

All ethical principles for medical research relating to human subjects were obligatory. The study was approved by the Scientific Research Committee of the School of Pharmacy and Deanship of Scientific Research at the University of Jordan approved the study. Further approval was obtained from the Jordan University Hospital Institutional Review Board (IRB) according to human and animal rights and Helsinki Declaration [9b]. Demographic data, as well as anthropometric measurements and lab tests, were obtained from each participant.

Consequently, adiposity, atherogenicity and blood indices were calculated as the following:

- Waist-to-hip ratio (WHR)=waist circumference (cm)÷ hip circumference (cm) [10];
- Waist-to-height ratio (WHtR)=waist circumference (cm)÷height (cm) [11];
- Conicity index (CI)=WC (cm)÷0.109* $\sqrt{\text{weight(kg)}\div\text{height(m)}}$ [12];
- Body adiposity index (BAI)= (HC (cm) / (height (m)^{1.5}))-18 [13];
- Lipid accumulation product (LAP) [14];
- Male LAP= (WC (cm)-65) × (TG (triglycerides) concentration (mM));
- Female LAP= (WC (cm) - 58) × (TG concentration (mM));
- Visceral adiposity index (VAI) [15];
- Male VAI=WC (cm)÷39.68+(1.88*BMI) *(TG concentration÷1.03)*(1.31÷HDL-C);
- Female VAI =WC (cm)÷36.58+(1.89*BMI) *(TG concentration÷0.81)*(1.52÷HDL-C);
- Atherogenic index of plasma (AIP)= Log10 (TG concentration/HDL-C) [16];

- Total cholesterol/HDL-C (TC/HDL-C) ratio = Total cholesterol ÷ HDL-C [17];
- LDL-C/HDL-C ratio =LDL-C ÷ HDL-C [18];
- Non-HDL-C=total cholesterol-HDL-C [18];
- Platelet-to lymphocyte ratio (PLR)= Platelets counts÷ Lymphocytes counts [19];
- Neutrophil-to-lymphocyte ratio (NLR)= Neutrophils counts ÷ Lymphocytes counts [19];
- Monocyte-to-lymphocyte ratio (MLR)= Monocytes counts ÷ Lymphocytes counts [20];

Statistical analysis

All study participants were organized accordingly to the study groups. Data were entered and tested through IBM SPSS®statistics 22 (SPSS, Inc., USA). Gender variation between the groups was analyzed utilizing the Chi-square test. Results were expressed as mean ±SD. The One-Way Analysis of Variance (ANOVA) test was used to contrast continuous dependent variables across the study groups. To assess the strength and direction of the association between continuous variables in MetS groups, which contain both normoglycemic and prediabetic MetS subjects, we used the Spearman's rank correlation coefficient.

Results

Demographic data

All study participants were Jordanians. Females were the majority of patients, representing 72.72% of all study population, and the gender was not distributed homogeneously between the three study groups but with no marked discrepancies between the 3 groups (Table 1). On the contrary, the age had vital variations between study groups ($P < 0.001$).

Clinical, adiposity, atherogenicity, and hematological indices

Both MetS groups (normoglycemic and preDM) had significantly higher values of DBP ($P < 0.001$), SBP ($P < 0.001$), TG ($P < 0.001$), and

Table 1: Comparison of anthropometric and clinical parameters, adiposity, atherogenicity, and hematological indices as well as metabolic risk biomarkers in study participants.

Gender						
Gender	Total Sample	Control Group	Normoglycemic-MetS Group	MetS-PreDM Group		#P-value
Female, N (%)	64 (72.72%)	22 (75.86%)	23 (79.3%)	19 (63.3%)		0.334
Male, N (%)	24 (26.1%)	7 (24.13%)	6 (20.70%)	11 (36.6%)		
Total	88 (100%)	29 (100%)	29 (100%)	30 (100%)		
Age						
						*P-value
Age (years, Mean ±SD)	49.72±1.17	44.39±2.05	49.07±2.00	55.68±1.51		<0.001
Clinical characteristics						
	Control, group N=29 Mean±SD#	MetS group, N=29 Mean±SD#	MetS-PreDM, group. N=30 Mean±SD#	P ¹ -value	P ² -value	P ³ -value
SBP (mmHg)	117.1±2.23	138.17±1.64	137.42±3.06	<0.001	<0.001	1
DBP (mmHg)	73.03±1.86	86.47±1.56	85.42±2.26	<0.001	<0.001	1
FBG (mg/dL)	85.31±1.40	88.53±1.72	112.57±3.45	1	<0.001	<0.001
A1C (%)	5.08±0.07	5.28±0.06	6.22±0.13	0.408	<0.001	<0.001
TG (mg/dL)	92.82±5.49	201.68±16.89	210.93±25.64	<0.001	<0.001	1
LDL-C (mg/dL)	129.65±6.07	143.87±6.12	142.21±7.23	0.380	0.519	1
HDL-C (mg/dL)	59.33±2.26	44.00±2.05	47.19±3.07	<0.001	0.003	1
TC (mg/dL)	195.80±6.26	208.60±7.32	218.03±8.40	0.673	0.105	1
Non-HDL-C (mg/dL)	136.46±6.96	164.59±6.35	170.84±7.86	0.019	0.003	1
Adiposity indices						
WC (cm)	87.23±1.81	107.1±1.96	107.66±2.30	<0.001	<0.001	1
HC (cm)	99.13±1.53	116.73±2.00	115.19±2.29	<0.001	<0.001	1
BMI (Kg/m²)	23.20±0.34	33.85±1.04	33.10±1.23	<0.001	<0.001	1
WHR	0.88±0.01	0.92±0.01	0.94±0.01	0.083	0.004	0.847
WHtR	0.54±0.01	0.67±0.01	0.67±0.02	<0.001	<0.001	1
CI	1.30±0.02	1.34±0.01	1.36±0.02	0.424	0.057	1
BAI	29.92±0.78	39.66±1.24	38.77±1.71	<0.001	<0.001	1
LAP	29.09±3.09	108.99±10.79	102.91±11.90	<0.001	<0.001	1
VAI	1.33±0.12	3.95±0.45	5.38±1.76	0.252	0.024	1
Atherogenicity indices						
AIP	0.19±0.04	0.63±0.05	0.62±0.07	<0.001	<0.001	1
TC/HDL-C	3.47±0.20	4.90±0.20	6.12±1.32	0.617	0.058	0.835
LDL-C/HDL-C	2.40±0.17	3.37±0.15	3.59±0.43	0.053	0.01	1
Non-HDL-C/HDL-C	2.48±0.20	3.90±0.20	5.16±1.32	0.621	0.051	0.773

Table 1: Continued

<i>Clinical characteristics</i>						
	Control, group N=29 Mean±SD#	MetS group, N=29 Mean±SD#	MetS-PreDM, group. N=30 Mean±SD#	P ¹ -value	P ² -value	P ³ -value
Hematological indices						
RDW-CV (%)	14.4±0.23	14.45±0.18	14.62±0.25	1	1	1
PLT count (× 10 ⁹ /L)	273.03±12.36	274.50±11.42	271.23±8.92	1	1	1
Monocytes%	5.63±0.28	5.31±0.24	5.40±0.24	1	1	1
Neutrophils%	57.39±1.12	57.39±1.59	59.82±1.72	1	0.755	0.769
Lymphocytes%	33.49±1.17	33.14±1.27	30.20±1.46	1	0.23	0.348
MLR	0.17±0.01	0.17±0.01	0.21±0.03	1	0.432	0.288
NLR	1.81±0.10	1.87±0.13	2.53±0.43	1	0.174	0.253
PLR	8.46±0.49	8.79±0.57	10.33±1.08	1	0.257	0.479
Metabolic risk biomarkers						
OCN (ng/mL)	23.3±3.19	28.13±1.22	26.02±3.2	<0.001	0.01	0.38
SIRT1 (ng/mL)	3.88±0.95	1.42±0.47	1.64±0.58	<0.001	<0.001	0.786

Note: P-value was obtained by ANOVA test. For gender we obtained the P-value using the Chi-Square test. Pairwise comparisons were done through Bonferroni adjustment.

P-value <0.05 are **bold**. P¹: normoglycemic MetS group versus control, P²: MetS-PreDM versus control, P³: MetS-PreDM versus MetS normoglycemic. AIP: atherogenicity index of plasma, BAI: body adiposity index, CI: conicity index, DBP: diastolic blood pressure, FPG: fasting plasma glucose, A1C: glycosylated hemoglobin A1C, HC: hip circumference, HDL-C: high density lipoprotein-cholesterol, LAP: lipid accumulation product, LDL-C/HDL-C: low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio, LDL-C: low density lipoprotein-cholesterol, MLR: monocyte-to-lymphocyte ratio, NLR: neutrophil-to-lymphocyte ratio, non-HDL-C/HDL: non high density lipoprotein-to-high density lipoprotein ratio, non-HDL-C: non-high density lipoprotein cholesterol, OCN: Osteocalcin, PLR: platelet-to-lymphocyte ratio. PLT: platelets, RDW: red cell width, SBP: systolic blood pressure, SIRT1: Sirtuin 1, TC/HDL-C: total cholesterol-to-high density lipoprotein cholesterol ratio, TC: total cholesterol, TG: triglycerides, WC: waist circumference, WHR: waist-to-hip ratio, WHtR: waist-to-height ratio.

non-HDL-C (P₁ = 0.019, P₂ = 0.003) than the control group and significantly lower values of HDL-C (p₁ < 0.001, p₂ = 0.003) in contrast to control group. However, in contrast to other clinical characteristics, the MetS-PreDM group presented significantly higher values of FPG and HbA1C than both normoglycemic MetS group and control group (P < 0.001) (Table 1). Also, both MetS groups (normoglycemic and PreDM) had a significantly higher value of adiposity indices, namely BMI, WC, HC, WHtR, BAI, and LAP than the control group (P < 0.001). Outstandingly, WHR and VAI had a significantly higher value in the MetS prediabetic group compared to controls (P < 0.05). As for hematologic parameters, all the parameters failed to

report any significant variability evidence in all the study groups (P > 0.05). Interestingly, in our atherogenicity indices, as shown in Table 1, both normoglycemic MetS and MetS-PreDM groups had significantly higher AIP in comparison to the control group (P < 0.001), meanwhile the LDL-C/HDL-C ratio was significantly higher in MetS-PreDM compared to the control group (P < 0.05).

SIRT1 and OCN plasma levels

OCN level (OCN; ng/mL) was significantly higher in normoglycemic MetS vs. both MetS-PreDM and controls (28.13±1.22 and 26.02±3.2 vs.

23.3±3.19, $P<0.001$ respectively). In contrast, the circulating level of SIRT1 (ng/mL) was lower in both normoglycemic and preDM MetS vs. healthy controls (1.42±0.47 and 1.64±0.58 vs. 3.88±0.95; $P<0.001$, respectively) (Table 1).

SIRT1 and OCN Correlations

As shown in Table 2, in the MetS pool of participants (N=59), no OCN-SIRT1 relation could be disclosed. No molecular metabolic risk biomarker was related to the subclinical inflammation indicator RDW-CV. With the exception of HC, OCN notably lacked positive interdependence with any of the adiposity indices. SIRT1 was associated with the LAP and VAI adiposity indices directly, but inversely with PLT in the same pool of MetS participants ($r_s=0.303$, $r_s=0.323$, $r_s=0.286$, $r_s=0.275$, $r_s=0.275$, $r_s=-0.314$, respectively, $P<0.05$). Substantially, SIRT1 was proportionally associated with the triad of TG, TG/HDL-C and AIP. Nevertheless, OCN did not relate to any of the lipid profile parameters (non-HDL-C inclusive) or any of their atherogenicity indices. OCN related reciprocally with FPG, A1C, monocytes, and its MLR ($r_s=0.266$, $r_s=-0.376$, $r_s=-0.317$, $r_s=-0.359$, $r_s=-0.309$, respectively, $P<0.05$). SIRT1 lacked marked relatedness with the glycemic control or any of blood indices;

The supplementary describes the relationships between clinical, adiposity, atherogenicity, and hematological parameters in the 59 MetS (Pre-DM and normoglycemic) recruits (Supplementary).

Discussion

In our heterogeneous pool of nondiabetic and prediabetic MetS recruits (n=59), metabolism-related adiposity, atherogenicity and blood indices, and molecular metabolic risk biomarkers SIRT1 and OCN were compared vs. the respective parameters in normoglycemic and lean controls. Further testing of putative relationships between indices and markers was also conducted in 59 participants from the MetS group. In a study by Calabrese et al. [21], the analysis of lymphocytes

revealed a significant reduction of the SIRT1 expression in T2DM patients in a striking dissimilarity to our study. Effectively western blot analysis of peripheral blood mononuclear cell's SIRT1 in MetS patients with the ATP III criteria by De Kreutzenberg et al. [22] revealed significantly lower levels of SIRT1 expression in MetS vs. non-MetS subjects. Our study also showed a novel finding that SIRT1 is associated with TG, LAP, VAI, TG/HDL-C ratio, AIP, and PLT in the pool of MetS participants.

Former studies illustrated that plasma OCN concentrations were reduced in diabetic patients, so evidently, OCN might be a hopeful predictive marker to estimate the T2DM risk. In a cross-sectional study by Bador et al. [23], the plasma concentration of OCN was compared between IDF defined-MetS participants that were segregated into diabetic and nondiabetic. Their study found a significant relationship between lower OCN plasma levels, and higher FPG and homeostasis model assessment of insulin resistance (HOMA-IR) in MetS patients with DM vs. those without DM. Compared to ours, these results showed that MetS-Pre-DM (unlike normoglycemic-MetS) subjects had substantially higher HbA1C and FPG values with lower OCN plasma levels. Also, the results of Bao et al. [24] were effectively comparable with our findings [24]. Study subjects were divided into four groups according to quartiles of serum osteocalcin levels: 25th percentile (less than 13.11 ng/mL), 25–50th percentile (13.12 - 16.17 ng/mL), 50–75th percentile (16.18 - 19.88 ng/mL) and 75th percentile (more than 19.89 ng/mL). Bao et al. revealed a decreasing trend in serum osteocalcin levels that accompanied increases in FPG, 2hPG, A1C, and HOMA-IR levels [24].

Conflicting results in the association of OCN with adiposity, hypertension, and dyslipidemia were delineated. Xu et al. [25] investigated the relationship between serum OCN levels and blood pressure in non-hypertensive and hypertensive groups from a Chinese population. The serum OCN level in their study was not associated with blood pressure in women nor with DBP in men, but it was associated significantly with SBP in men [25]. However, this association disappeared after adjustment for BMI, WC, blood

Table 2: Osteocalcin and Sirtuin 1 correlations in MetS (both normoglycemic and PreDM) participants (N=59).

Clinical parameters												
		SBP (mm Hg)	DBP (mm Hg)	FBG (mg/dL)	A1C (%)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TC (mg/dL)	Non-HDL-C (mg/dL)		
Normoglycemic and PreDM MetS	OCN (ng/mL)	R	0.227	0.148	-0.376	-0.317	0.118	0.138	0.128	0.113		
		P-value	0.084	0.263	0.003	0.014	0.372	0.298	0.336	0.395		
	SIRT1 (ng/mL)	R	0.138	0.173	0.062	0.136	0.303	-0.087	-0.011	0.052		
		P-value	0.297	0.191	0.639	0.303	0.020	0.510	0.933	0.696		
Adiposity indices												
		WC (cm)	HC (cm)	CI	BMI (Kg/m ²)	BAI	WHR	WHtR	LAP	VAI		
Normoglycemic and PreDM MetS	OCN (ng/mL)	R	0.166	0.266	0.147	0.211	0.228	0.202	0.250	0.068		
		P-value	0.208	0.042	0.267	0.108	0.082	0.125	0.057	0.608		
	SIRT1 (ng/mL)	R	0.119	0.123	0.160	0.051	0.085	0.109	0.323	0.286		
		P-value	0.369	0.354	0.226	0.704	0.522	0.413	0.013	0.028		
Atherogenicity indices												
		OCN (ng/ mL)	SIRT1 (ng/ mL)	Non-HDL-C/ HDL-C ratio	TG/HDL-C ratio	TC/HDL-C ratio	LDL-C/HDL-C ratio					
Normoglycemic and PreDM MetS	OCN (ng/mL)	R	1.000	0.105	0.002	0.056	0.003	-0.060		0.056		
		P-value		0.429	0.985	0.674	0.981	0.649		0.674		
	SIRT1 (ng/mL)	R	0.105	1.000	0.076	0.275	0.075	-0.008		0.275		
		P-value	0.429		0.566	0.035	0.572	0.952		0.035		
Hematological indices												
		RDW-CV %	PLT count	Monocytes %	Neutrophils %	Lymphocytes %	MLR	NLR	PLR			
Normoglycemic and PreDM MetS	OCN (ng/mL)	R	0.160	0.048	-0.359	0.077	0.012	0.039	-0.063			
		P-value	0.227	0.721	0.005	0.562	0.930	0.772	0.635			
	SIRT1 (ng/mL)	R	-0.034	-0.314	-0.204	0.080	-0.036	0.068	-0.095			
		P-value	0.801	0.015	0.120	0.544	0.787	0.608	0.473			

Spearman's correlation coefficient * was used, R=0.1-0.29 small relationship, R=0.3-0.49 moderate relationship, R>0.5 high relationship. AIP: atherogenicity index of plasma, BAI: body adiposity index, CI: conicity index, DBP: diastolic blood pressure, FPG: fasting plasma glucose, A1C: glycosylated hemoglobin A1C, HC: hip circumference, HDL-C: high density lipoprotein-cholesterol, LAP: lipid accumulation product, LDL-C/HDL-C: low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio, LDL-C: low density lipoprotein-cholesterol, MLR: monocyte-to-lymphocyte ratio, NLR: neutrophil-to-lymphocyte ratio, non-HDL-C/HDL-C: non high density lipoprotein-to-high density lipoprotein ratio, non-HDL-C: non-high density lipoprotein cholesterol, OCN: Osteocalcin, PLR: platelet-to-lymphocyte ratio, PLT: platelets, RDW: red cell width, SBP: systolic blood pressure, SIRT1: Sirtuin 1, TC/HDL-C: total cholesterol-to-high density lipoprotein cholesterol ratio, TC: total cholesterol, TG: triglycerides, WC: waist circumference, WHR: waist-to-hip ratio, WHtR: waist-to-height ratio.

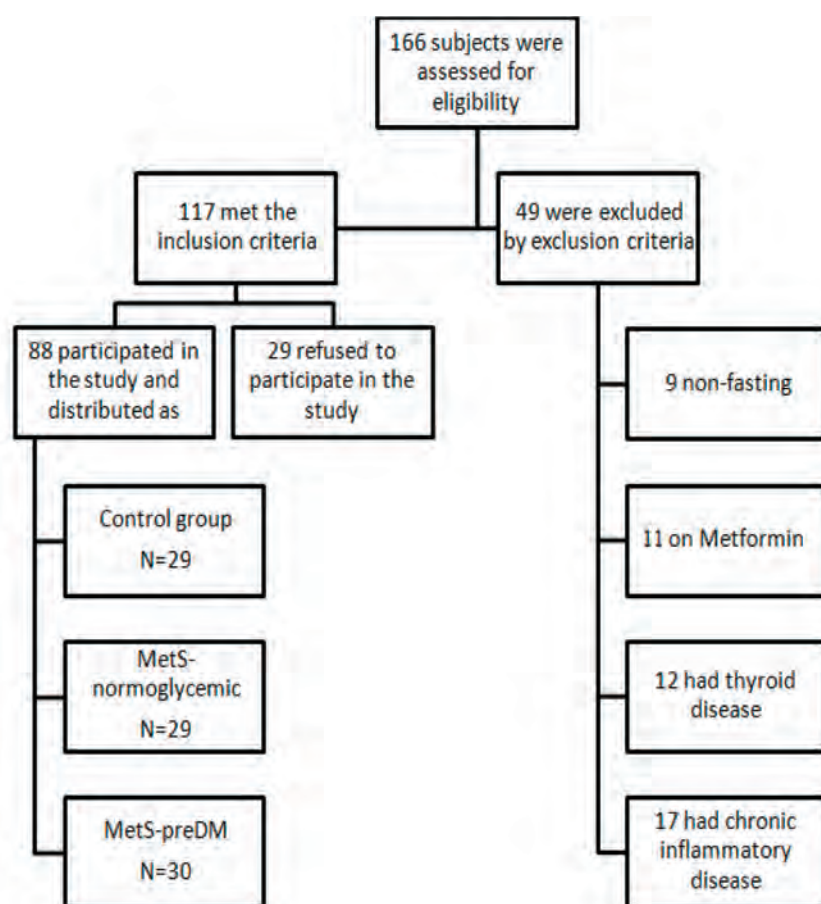


Figure 1: Recruitment process flow chart.

glucose, and HOMA-IR. The opposite finding was observed in the study of Magalhães et al. [9]. There was a significant association between serum OCN levels and SBP, BMI, WC, and FBG in a Brazilian population with and without MetS.

Similarly, the study results of Bao et al. indicated that serum OCN levels correlated with all the anthropometric indices of obesity: SBP, FPG, 2-h plasma glucose (2hPG), HbA1C, HOMA-IR, TC, TG, and free fatty acids [24]. In accordance with our study, the serum OCN concentrations were not associated with SBP, DBP, TG, LDL-C, HDL-C, TC, WC, or BMI in MetS patients. However, they were correlated with FBG, consistent with the results of Bador et al. [23]. Moreover, none of the above studies examined the correlation of serum OCN levels with atherogenicity and hematological indices. Nevertheless, our results revealed that plasma OCN concentrations correlated with MLR and monocytes. Regarding the limitations of our study, it must be noted that no causality relationship may be concluded with the cross-sectional study

design, which includes a single time point for sampling biomarkers and subsequent determination and that recruiting a larger sample size was hindered owing to financial restrictions.

Conclusions

We found that OCN levels were significantly higher in MetS vs. healthy non-MetS controls; the circulating level of SIRT1 was lower in both groups: normoglycemic and PreDM-MetS vs. healthy controls. Also,

WC, HC, BMI, WHtR, BAI, LAP, and AIP were significantly higher in both MetS (non- and PreDM) groups vs. controls. The LDL-C/HDL-C ratio, VAI, and WHR were greater in MetS-PreDM (but not normoglycemic MetS) vs. lean recruits. OCN correlated positively with HC, but reciprocally with FPG, A1C, monocytes, and MLR. SIRT1 associated proportionally with TG, TG/HDL-C ratio, and AIP, LAP, and VAI, but inversely with PLT in the same pool of MetS participants.

However, except for FPG and HbA1C, no further intergroup variation could be identified between the MetS groups. Also, no OCN-SIRT1 relation could be detected in the 59 MetS participants (normoglycemic and PreDM).

No biomarker (OCN/SIRT1) correlated with SBP, DBP, LDL-C, HDL-C, TC, non-HDL-C, WC, CI, BMI, BAI, WHR, WHtR, non-HDL-C/HDL-C ratio, TC/HDL-C ratio, LDL-C/HDL-C ratio, RDW-CV%, neutrophils, lymphocytes, NLR, and PLR.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary

It demonstrates correlations between clinical, adiposity, atherogenicity, and hematological parameters in MetS group as the following:

Age: Age correlated positively with WC, CI, WHtR, FBG, and A1C, but negatively with DBP.
Both A1C and FBG correlated positively with each other.

Adiposity indices

Weight: Weight associated positively with WC, HC, BMI, WHtR, LAP, and RDW-CV%.
Height: Height correlated inversely with BMI, BAI, and WHtR.
WC: WC associated positively with weight, HC, CI, BMI, BAI, WHR, WHtR, and LAP.
HC: HC correlated disproportionally with WHR, but positively with weight, WC, BMI, BAI, WHtR, LAP, and RDW-CV%.
CI: CI associated positively with WC, WHR, and WHtR.
BAI: BAI associated positively with HDL-C, WHtR and RDW-CV%.
WHtR: WHtR correlated negatively with height but positively with LAP, BMI and HDL-C.

Atherogenicity indices

TG: TG associated positively with LAP, TC, Non-HDL-C, Non-HDL-C/HDL-C ratio, VAI, TC/HDL-C ratio, LDL-C/HDL-C ratio, TG/HDL-C ratio, and AIP.
LAP: LAP associated positively with TC, Non-HDL-C, Non-HDL-C/HDL-C ratio, VAI, TC/HDL-C ratio, LDL-C/HDL-C ratio, TG/HDL-C ratio, and AIP.
LDL-C: LDL-C correlated positively with Non-HDL-C/HDL-C ratio, TC/HDL-C ratio, LDL-C/HDL-C ratio, and PLT.
HDL-C: HDL-C correlated inversely with Non-HDL-C/HDL-C ratio, VAI, TC/HDL-C ratio, LDL-C/HDL-C ratio, TG/HDL-C ratio, and AIP, but positively with PLT.
TC: TC correlated positively with LDL-C, HDL-C, Non-HDL-C, Non-HDL-C/HDL-C ratio, TC/HDL-C ratio, LDL-C/HDL-C ratio, and PLT.
Non-HDL-C and Non-HDL-C/HDL-C ratio: they associated positively with each other and with LDL-C, VAI, TC/HDL-C ratio, LDL-C/HDL-C ratio, TG/HDL-C ratio, and AIP.
VAI: VAI correlated positively with TC/HDL-C ratio, LDL-C/HDL-C ratio, TG/HDL-C ratio, and AIP.
TC/HDL-C ratio and LDL-C/HDL-C ratio: both they positively correlated with each other.

Blood indices

MLR, NLR, and PLR: They correlated negatively with lymphocytes and positively with each other and with neutrophils.

Supplementary correlations of adiposity, clinical, atherogenicity, and hematological parameters in the MetS groups (Both normoglycemic and pre-diabetic) participants (N=59).

		Age (years)	Weight (kg)	Height (cm)	WC (cm)	HC (cm)	CI	BMI (kg/m ²)	BAI	WHR
Age (years)	R	1.000	-0.067	-0.332	0.281	0.059	0.438	0.010	0.232	0.197
	P-value		0.612	0.010	0.031	0.658	0.001	0.938	0.077	0.134
Weight (kg)	R	-0.067	1.000	0.398	0.720	0.647	-0.164	0.701	0.223	-0.015
	P-value	0.612		0.002	0.000	0.000	0.213	0.000	0.089	0.909
Height (cm)	R	-0.332	0.398	1.000	-0.071	-0.147	-0.226	-0.269	-0.714	0.105
	P-value	0.010	0.002		0.591	0.267	0.085	0.040	0.000	0.430
WC (cm)	R	0.281	0.720	-0.071	1.000	0.771	0.416	0.779	0.570	0.269
	P-value	0.031	0.000	0.591		0.000	0.001	0.000	0.000	0.039
HC (cm)	R	0.059	0.647	-0.147	0.771	1.000	0.113	0.805	0.750	-0.313
	P-value	0.658	0.000	0.267	0.000		0.393	0.000	0.000	0.016
CI	R	0.438	-0.164	-0.226	0.416	0.113	1.000	-0.056	0.174	0.590
	P-value	0.001	0.213	0.085	0.001	0.393		0.676	0.187	0.000
BMI (kg/m ²)	R	0.010	0.701	-0.269	0.779	0.805	-0.056	1.000	0.765	-0.106
	P-value	0.938	0.000	0.040	0.000	0.000	0.676		0.000	0.423
BAI	R	0.232	0.223	-0.714	0.570	0.750	0.174	0.765	1.000	-0.298
	P-value	0.077	0.089	0.000	0.000	0.000	0.187	0.000		0.022
WHR	R	0.197	-0.015	0.105	0.269	-0.313	0.590	-0.106	-0.298	1.000
	P-value	0.134	0.909	0.430	0.039	0.016	0.000	0.423	0.022	
WHtR	R	0.329	0.387	-0.563	0.832	0.710	0.421	0.816	0.868	0.127
	P-value	0.011	0.002	0.000	0.000	0.000	0.001	0.000	0.000	0.338
SBP (mmHg)	R	-0.136	0.082	-0.026	0.075	0.060	0.088	0.092	0.057	0.048
	P-value	0.304	0.539	0.844	0.572	0.650	0.509	0.489	0.671	0.716
DBP (mmHg)	R	-0.290	-0.031	0.165	-0.157	-0.100	-0.053	-0.149	-0.188	0.038
	P-value	0.026	0.815	0.211	0.236	0.452	0.692	0.259	0.153	0.775
FBG (mg/dL)	R	0.268	-0.155	0.064	-0.028	-0.085	0.183	-0.176	-0.064	-0.019
	P-value	0.040	0.240	0.633	0.836	0.522	0.166	0.182	0.630	0.889
A1C%	R	0.337	0.046	0.010	0.193	0.040	0.177	0.039	0.028	0.088
	P-value	0.009	0.727	0.942	0.144	0.765	0.180	0.767	0.836	0.510
TG (mg/dL)	R	-0.083	0.053	0.087	-0.054	-0.011	-0.118	-0.025	-0.039	-0.038
	P-value	0.530	0.691	0.513	0.684	0.933	0.375	0.850	0.767	0.775
LAP	R	0.007	0.303	-0.053	0.352	0.334	0.098	0.339	0.284	0.073
	P-value	0.959	0.020	0.692	0.006	0.010	0.458	0.009	0.029	0.584
LDL-C mg/dL	R	0.131	-0.161	-0.342	-0.012	0.006	0.029	0.051	0.243	-0.075
	P-value	0.321	0.224	0.008	0.931	0.967	0.829	0.699	0.064	0.573
HDL-C (mg/dL)	R	0.034	-0.141	-0.412	0.100	0.175	0.117	0.183	0.354	-0.080
	P-value	0.796	0.288	0.001	0.450	0.184	0.378	0.165	0.006	0.549
TC (mg/dL)	R	0.074	-0.201	-0.325	-0.099	-0.078	-0.013	-0.005	0.177	-0.053
	P-value	0.575	0.126	0.012	0.458	0.559	0.919	0.972	0.180	0.691

		Age (years)	Weight (kg)	Height (cm)	WC (cm)	HC (cm)	CI	BMI (kg/m ²)	BAI	WHR
Non-HDL-C (mg/dL) RDW-CV%	R	0.109	-0.195	-0.317	-0.133	-0.131	-0.069	-0.011	0.148	-0.037
	P-value	0.413	0.139	0.014	0.316	0.321	0.601	0.935	0.263	0.779
	R	-0.031	0.276	-0.122	0.249	0.370	-0.139	0.424	0.319	-0.129
	P-value	0.817	0.034	0.355	0.058	0.004	0.293	0.001	0.014	0.332

		WHtR	SBP (mmHg)	DBP (mmHg)	FBG (mg/ dL)	A1C %	TG (mg/ dL)	LAP	LDL-C (mg/dL)	HDL (mg/dL)
Age (years)	R	0.329	-0.136	-0.290	0.268	0.337	-0.083	0.007	0.131	0.034
	P-value	0.011	0.304	0.026	0.040	0.009	0.530	0.959	0.321	0.796
Weight (kg)	R	0.387	0.082	-0.031	-0.155	0.046	0.053	0.303	-0.161	-0.141
	P-value	0.002	0.539	0.815	0.240	0.727	0.691	0.020	0.224	0.288
Height (cm)	R	-0.563	-0.026	0.165	0.064	0.010	0.087	-0.053	-0.342	-0.412
	P-value	0.000	0.844	0.211	0.633	0.942	0.513	0.692	0.008	0.001
WC (cm)	R	0.832	0.075	-0.157	-0.028	0.193	-0.054	0.352	-0.012	0.100
	P-value	0.000	0.572	0.236	0.836	0.144	0.684	0.006	0.931	0.450
HC (cm)	R	0.710	0.060	-0.100	-0.085	0.040	-0.011	0.334	0.006	0.175
	P-value	0.000	0.650	0.452	0.522	0.765	0.933	0.010	0.967	0.184
CI	R	0.421	0.088	-0.053	0.183	0.177	-0.118	0.098	0.029	0.117
	P-value	0.001	0.509	0.692	0.166	0.180	0.375	0.458	0.829	0.378
BMI (kg/m ²)	R	0.816	0.092	-0.149	-0.176	0.039	-0.025	0.339	0.051	0.183
	P-value	0.000	0.489	0.259	0.182	0.767	0.850	0.009	0.699	0.165
BAI	R	0.868	0.057	-0.188	-0.064	0.028	-0.039	0.284	0.243	0.354
	P-value	0.000	0.671	0.153	0.630	0.836	0.767	0.029	0.064	0.006
WHR	R	0.127	0.048	0.038	-0.019	0.088	-0.038	0.073	-0.075	-0.080
	P-value	0.338	0.716	0.775	0.889	0.510	0.775	0.584	0.573	0.549
WHtR	R	1.000	0.109	-0.179	-0.060	0.148	-0.049	0.346	0.158	0.283
	P-value		0.413	0.175	0.653	0.264	0.713	0.007	0.231	0.030
SBP (mmHg)	R	0.109	1.000	0.692	-0.086	0.053	-0.049	-0.013	-0.015	0.180
	P-value	0.413		0.000	0.519	0.691	0.711	0.923	0.910	0.172
DBP (mmHg)	R	-0.179	0.692	1.000	-0.139	-0.007	0.115	0.032	-0.007	0.068
	P-value	0.175	0.000		0.294	0.960	0.385	0.808	0.959	0.607
FBG (mg/dL)	R	-0.060	-0.086	-0.139	1.000	0.736	-0.055	-0.145	-0.136	-0.046
	P-value	0.653	0.519	0.294		0.000	0.678	0.272	0.303	0.730
A1C %	R	0.148	0.053	-0.007	0.736	1.000	-0.062	-0.087	-0.023	0.064
	P-value	0.264	0.691	0.960	0.000		0.642	0.515	0.864	0.629
TG (mg/dL)	R	-0.049	-0.049	0.115	-0.055	-0.062	1.000	0.865	0.104	-0.335
	P-value	0.713	0.711	0.385	0.678	0.642		0.000	0.431	0.009
LAP	R	0.346	-0.013	0.032	-0.145	-0.087	0.865	1.000	0.134	-0.224
	P-value	0.007	0.923	0.808	0.272	0.515	0.000		0.311	0.088

		WHtR	SBP (mmHg)	DBP (mmHg)	FBG (mg/ dL)	A1C %	TG (mg/ dL)	LAP	LDL-C (mg/dL)	HDL (mg/dL)
LDL-C mg/dL	R	0.158	-0.015	-0.007	-0.136	-0.023	0.104	0.134	1.000	0.424
	P-value	0.231	0.910	0.959	0.303	0.864	0.431	0.311		0.001
HDL-C (mg/dL)	R	0.283	0.180	0.068	-0.046	0.064	-0.335	-0.224	0.424	1.000
	P-value	0.030	0.172	0.607	0.730	0.629	0.009	0.088	0.001	
TC (mg/dL)	R	0.086	-0.036	-0.001	-0.044	-0.001	0.312	0.300	0.842	0.453
	P-value	0.518	0.785	0.991	0.740	0.991	0.016	0.021	0.000	0.000
Non- HDL-C (mg/dL)	R	0.057	-0.058	-0.012	-0.124	-0.099	0.426	0.401	0.846	0.197
	P-value	0.668	0.660	0.927	0.348	0.456	0.001	0.002	0.000	0.135
Non- HDL-C/ HDL-C ratio	R	-0.153	-0.191	-0.041	-0.040	-0.105	0.647	0.506	0.268	-0.657
	P-value	0.246	0.148	0.759	0.762	0.427	0.000	0.000	0.040	0.000
VAI	R	-0.041	-0.130	0.019	-0.093	-0.126	0.897	0.803	-0.007	-0.607
	P-value	0.760	0.326	0.885	0.482	0.340	0.000	0.000	0.960	0.000
TC/ HDL-C	R	-0.151	-0.190	-0.041	-0.039	-0.105	0.649	0.508	0.267	-0.658
	P-value	0.252	0.149	0.759	0.770	0.430	0.000	0.000	0.041	0.000
LDL-C/ HDL-C	R	-0.177	-0.189	-0.020	-0.020	-0.037	0.475	0.343	0.449	-0.548
	P-value	0.180	0.151	0.880	0.879	0.779	0.000	0.008	0.000	0.000
TG/ HDL-C ratio	R	-0.168	-0.111	0.063	-0.061	-0.107	0.886	0.723	-0.061	-0.686
	P-value	0.203	0.404	0.637	0.646	0.420	0.000	0.000	0.645	0.000
AIP	R	-0.168	-0.111	0.063	-0.061	-0.107	0.886	0.723	-0.061	-0.686
	P-value	0.203	0.404	0.637	0.646	0.420	0.000	0.000	0.645	0.000
RDW- CV%	R	0.248	-0.051	-0.173	-0.045	0.019	-0.041	0.107	-0.091	0.073
	P-value	0.058	0.700	0.189	0.734	0.886	0.756	0.418	0.492	0.584
PLT count ($\times 10^9/L$)	R	-0.092	0.116	0.025	-0.110	-0.164	-0.059	-0.089	0.276	0.321
	P-value	0.490	0.380	0.851	0.406	0.214	0.655	0.504	0.035	0.013
Monocytes %	R	-0.173	-0.277	-0.264	0.084	-0.042	-0.111	-0.195	-0.119	-0.201
	P-value	0.191	0.034	0.043	0.527	0.751	0.403	0.138	0.368	0.126

		TC (mg/dL)	Non-HDL (mg/dL)	Non- HDL-C/ HDL-C ratio	VAI	TC/ HDL-C ratio	LDL-C/ HDL-C ratio	TG/ HDL-C	AIP	RDW- CV%
Age (years)	R	0.074	0.109	0.062	0.008	0.065	0.122	-0.043	-0.043	-0.031
	P-value	0.575	0.413	0.639	0.952	0.627	0.359	0.746	0.746	0.817
Weight (kg)	R	-0.201	-0.195	-0.013	0.027	-0.016	-0.070	0.073	0.073	0.276
	P-value	0.126	0.139	0.925	0.840	0.905	0.597	0.581	0.581	0.034
Height (cm)	R	-0.325	-0.317	0.103	0.092	0.099	0.099	0.224	0.224	-0.122
	P-value	0.012	0.014	0.440	0.490	0.454	0.457	0.089	0.089	0.355
WC (cm)	R	-0.099	-0.133	-0.153	-0.032	-0.153	-0.171	-0.103	-0.103	0.249
	P-value	0.458	0.316	0.246	0.811	0.246	0.195	0.439	0.439	0.058
HC (cm)	R	-0.078	-0.131	-0.208	-0.022	-0.208	-0.223	-0.102	-0.102	0.370
	P-value	0.559	0.321	0.114	0.868	0.114	0.089	0.441	0.441	0.004
CI	R	-0.013	-0.069	-0.161	0.000	-0.159	-0.109	-0.123	-0.123	-0.139
	P-value	0.919	0.601	0.223	0.998	0.229	0.410	0.354	0.354	0.293
BMI (kg/m²)	R	-0.005	-0.011	-0.131	-0.071	-0.132	-0.189	-0.123	-0.123	0.424
	P-value	0.972	0.935	0.323	0.591	0.320	0.151	0.352	0.352	0.001
BAI	R	0.177	0.148	-0.147	-0.058	-0.145	-0.160	-0.186	-0.186	0.319
	P-value	0.180	0.263	0.265	0.660	0.272	0.226	0.159	0.159	0.014
TG (mg/dL)	R	0.312	0.426	0.647	0.897	0.649	0.475	0.886	0.886	-0.041
	P-value	0.016	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.756
LAP	R	0.300	0.401	0.506	0.803	0.508	0.343	0.723	0.723	0.107
	P-value	0.021	0.002	0.000	0.000	0.000	0.008	0.000	0.000	0.418
LDL-C mg/dL	R	0.842	0.846	0.268	-0.007	0.267	0.449	-0.061	-0.061	-0.091
	P-value	0.000	0.000	0.040	0.960	0.041	0.000	0.645	0.645	0.492
HDL-C (mg/dL)	R	0.453	0.197	-0.657	-0.607	-0.658	-0.548	-0.686	-0.686	0.073
	P-value	0.000	0.135	0.000	0.000	0.000	0.000	0.000	0.000	0.584
TC (mg/dL)	R	1.000	0.930	0.259	0.084	0.258	0.289	0.039	0.039	-0.058
	P-value		0.000	0.048	0.526	0.049	0.027	0.772	0.772	0.665
Non- HDL-C (mg/dL)	R	0.930	1.000	0.524	0.296	0.523	0.540	0.259	0.259	-0.063
	P-value	0.000		0.000	0.023	0.000	0.000	0.047	0.047	0.638
Non HDL-C/ HDL-C ratio	R	0.259	0.524	1.000	0.779	1.000	0.929	0.823	0.823	-0.088
	P-value	0.048	0.000		0.000	0.000	0.000	0.000	0.000	0.507
VAI	R	0.084	0.296	0.779	1.000	0.781	0.630	0.963	0.963	-0.013
	P-value	0.526	0.023	0.000		0.000	0.000	0.000	0.000	0.922
TC/ HDL-C	R	0.258	0.523	1.000	0.781	1.000	0.929	0.824	0.824	-0.088
	P-value	0.049	0.000	0.000	0.000		0.000	0.000	0.000	0.508
LDL-C/ HDL-C	R	0.289	0.540	0.929	0.630	0.929	1.000	0.660	0.660	-0.151
	P-value	0.027	0.000	0.000	0.000	0.000		0.000	0.000	0.253

		TC (mg/dL)	Non-HDL (mg/dL)	Non- HDL-C/ HDL-C ratio	VAI	TC/ HDL-C ratio	LDL-C/ HDL-C ratio	TG/ HDL-C	AIP	RDW- CV%
TG/ HDL-C ratio	R	0.039	0.259	0.823	0.963	0.824	0.660	1.000	1.000	-0.061
	P-value	0.772	0.047	0.000	0.000	0.000	0.000			0.648
AIP	R	0.039	0.259	0.823	0.963	0.824	0.660	1.000	1.000	-0.061
	P-value	0.772	0.047	0.000	0.000	0.000	0.000			0.648
RDW- CV%	R	-0.058	-0.063	-0.088	-0.013	-0.088	-0.151	-0.061	-0.061	1.000
	P-value	0.665	0.638	0.507	0.922	0.508	0.253	0.648	0.648	
PLT count ($\times 10^9/L$)	R	0.329	0.218	-0.123	-0.183	-0.126	-0.085	-0.189	-0.189	0.015
	P-value	0.011	0.097	0.353	0.166	0.342	0.521	0.151	0.151	0.910

		PLT count	Monocytes %	Neutrophils %	Lymphocytes %	MLR	NLR	PLR	OCN (ng/mL)	SIRT1 (ng/mL)
HC (cm)	R	0.068	-0.194	0.052	-0.011	-0.150	0.042	0.055	0.266	0.123
	P-value	0.607	0.140	0.694	0.935	0.255	0.751	0.678	0.042	0.354
FBG (mg/dL)	R	-0.110	0.084	0.005	-0.035	0.059	0.026	0.007	-0.376	0.062
	P-value	0.406	0.527	0.968	0.793	0.657	0.843	0.958	0.003	0.639
A1C %	R	-0.164	-0.042	0.083	-0.129	0.005	0.123	0.002	-0.317	0.136
	P-value	0.214	0.751	0.530	0.330	0.969	0.353	0.986	0.014	0.303
TG (mg/dL)	R	-0.059	-0.111	0.153	-0.151	-0.003	0.173	0.090	0.118	0.303
	P-value	0.655	0.403	0.247	0.253	0.979	0.190	0.499	0.372	0.020
LAP	R	-0.089	-0.195	0.083	-0.052	-0.127	0.086	0.007	0.250	0.323
	P-value	0.504	0.138	0.532	0.693	0.336	0.518	0.961	0.057	0.013
LDL-C mg/dL	R	0.276	-0.119	-0.197	0.183	-0.235	-0.187	-0.022	0.078	-0.083
	P-value	0.035	0.368	0.135	0.165	0.073	0.157	0.867	0.558	0.531
HDL-C (mg/dL)	R	0.321	-0.201	0.163	-0.128	-0.069	0.134	0.240	0.138	-0.087
	P-value	0.013	0.126	0.216	0.336	0.601	0.313	0.067	0.298	0.510
TC (mg/dL)	R	0.329	-0.124	-0.079	0.066	-0.170	-0.072	0.082	0.128	-0.011
	P-value	0.011	0.348	0.551	0.618	0.197	0.585	0.539	0.336	0.933
VAI	R	-0.183	-0.051	0.045	-0.034	-0.033	0.063	-0.043	0.068	0.286
	P-value	0.166	0.701	0.737	0.798	0.805	0.634	0.748	0.608	0.028
TG/HDL-C ratio	R	-0.189	-0.014	0.040	-0.054	-0.002	0.071	-0.042	0.056	0.275
	P-value	0.151	0.918	0.763	0.686	0.987	0.595	0.750	0.674	0.035
AIP	R	-0.189	-0.014	0.040	-0.054	-0.002	0.071	-0.042	0.056	0.275
	P-value	0.151	0.918	0.763	0.686	0.987	0.595	0.750	0.674	0.035
RDW-CV%	R	0.015	-0.096	0.167	-0.139	0.046	0.152	0.225	0.160	-0.034
	P-value	0.910	0.469	0.207	0.295	0.731	0.250	0.087	0.227	0.801

		PLT count	Monocytes %	Neutrophils %	Lymphocytes %	MLR	NLR	PLR	OCN (ng/mL)	SIRT1 (ng/mL)
PLT count ($\times 10^9/L$)	R	1.000	0.148	0.060	-0.141	0.177	0.109	0.611	0.048	-0.314
	P-value		0.265	0.651	0.288	0.180	0.410	0.000	0.721	0.015
Monocytes %	R	0.148	1.000	-.273	0.037	0.704	-0.121	0.067	-0.359	-0.204
	P-value	0.265		0.037	0.783	0.000	0.360	0.616	0.005	0.120
Neutrophils %	R	0.060	-0.273	1.000	-0.928	0.416	0.972	0.735	0.077	0.080
	P-value	0.651	0.037		0.000	0.001	0.000	0.000	0.562	0.544
Lymphocytes %	R	-0.141	0.037	-0.928	1.000	-0.635	-0.983	-0.824	0.012	-0.036
	P-value	0.288	0.783	0.000		0.000	0.000	0.000	0.930	0.787
MLR	R	0.177	0.704	0.416	-0.635	1.000	0.555	0.612	-0.309	-0.131
	P-value	0.180	0.000	0.001	0.000		0.000	0.000	0.017	0.323
NLR	R	0.109	-0.121	0.972	-0.983	0.555	1.000	0.796	0.039	0.068
	P-value	0.410	0.360	0.000	0.000	0.000		0.000	0.772	0.608
PLR	R	0.611	0.067	0.735	-0.824	0.612	0.796	1.000	-0.063	-0.095
	P-value	0.000	0.616	0.000	0.000	0.000	0.000		0.635	0.473

Spearman's correlation coefficient (R) was used, R=0.1-.0.29 small relationship, R=0.3-0.49 moderate relationship, R>0.5 high relationship. WC (cm), HC (cm), BMI (Kg/m²), A1c (%), TG (mg/dL), LDL-C (mg/dL), HDL-C (mg/dL), MPV (fL), RDW-CV (%), OCN (ng/mL), SIRT1 (ng/mL). WC: waist circumference, HC: hip circumference, WHR: waist-to-hip ratio, WHtR: waist-to-height ratio, CI: conicity index, BAI : body adiposity index, A1c: glycated hemoglobin A1C, TG: triglyceride, LDL-C: low density lipoprotein-cholesterol, HDL-C: high density lipoprotein-cholesterol, RDW: red cell distribution width, MPV: mean platelet volume, MLR: monocyte-to-lymphocyte ratio, NLR: neutrophil-to-lymphocyte ratio, PLR: platelet-to-lymphocyte ratio, AIP: atherogenicity index of plasma, TC/HDL-C: total cholesterol-to-high density lipoprotein cholesterol ratio, LDL-C/HDL-C: low density lipoprotein cholesterol-to-high density lipoprotein cholesterol ratio.

Original Research

The Role of Vitamin D in Treatment of Polycystic Ovary Syndrome Depending on Phenotype

Halyna Suslyk, Alina Urbanovych*, Orysia Lishchuk, Krystyna Kozlovska

Department of Endocrinology, Danylo Halytsky Lviv National Medical University, Ukraine

*Correspondence to: Alina Urbanovych, MD, PhD, Department of endocrinology, Lviv National Medical University; 79010, Lviv, Pekarska street, 69, Ukraine. E-mail: alinaur@dr.com. Phone: +380322764673

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Abstract

The goal of the study is to explore the changes of vitamin D, hormonal indices, fat, and carbohydrate metabolism in the blood of women with polycystic ovary syndrome (PCOS), as well as to define the appropriateness of prescribing cholecalciferol in the complex treatment of this pathology. To achieve this goal, we have examined 80 women, aged 18-35 years, with PCOS and underlying overweight and obesity, who were divided into four phenotype groups, according to the consensus of the PCOS Workshop Group in Amsterdam (2011). All women underwent a general medical examination, having measured their anthropometric parameters, body mass index (BMI) and clinical manifestations of hyperandrogenemia. Their serum was checked for the content of 25-hydroxyvitamin D (25(OH)D), LH, FSH, progesterone, estradiol, androstenedione, dehydroepiandrosterone sulfate, 17-hydroxyprogesterone, anti-Müllerian hormone, insulin, and leptin. The study results found a decrease in vitamin D in the blood of women with PCOS in all clinical phenotypes, which lowered with the progression of obesity. Cholecalciferol deficiency was associated with insulin resistance, hyperinsulinemia and hyperleptinemia. The correlation between the vitamin D content and hormonal indices in women with PCOS showed an inverse correlation between 25(OH)D and AMH levels, positive between insulin and 25(OH)D, and negative between vitamin D level and hirsutism score. Prescription of vitamin D in the complex treatment of women with PCOS leads to stabilization of hormones, decrease of HA signs, normalization of the menstrual cycle and restored ovulation.

Keywords: Hyperandrogenism, Insulin resistance, Obesity, Polycystic ovarian syndrome, Vitamin D deficiency.

Background and Aims

For many years polycystic ovary syndrome (PCOS) has been one of the most important medical and social issues since it is a significant component in the structure of diseases that cause female infertility. PCOS causes more than half of all cases of endocrine infertility (50-75%) and approximately 20-22% of cases of infertility in general [1]. However, the impact of PCOS is not limited to infertility only. Today, the syndrome catches the attention not only as causing the pathology of the reproductive system but also by impairing the functioning of almost all organs and systems of the body. The metabolic components of the syndrome increase the risk of diabetes mellitus type 2 (DMTII)

ten times, cardiovascular pathology, as well as cancer, seven times, which, of course, affects not only the quality but also the life expectancy of a woman [2, 3]. Thus, in the final report of the US National Institutes of Health on PCOS, this pathology was identified as one of the priorities in maintaining the health and quality of life of women, requiring interdisciplinary research to study the mechanisms of ovarian, hypothalamic-pituitary-adrenal, and metabolic dysfunction, defining the prevalence of PCOS phenotypes and their contribution to the risk of cardiovascular pathology, DMTII, and cancer [4, 5, 6].

PCOS is diagnosed in 36% of women of fertile age [7, 8]. The frequency of PCOS diagnostics is quite variable due to its heterogeneous, clinical, and endocrinological



manifestations. The incidence of PCOS is much higher among women with overweight and obesity [9].

The main clinical manifestations of PCOS are: menstrual dysfunction with chronic anovulation and androgen-dependent dermatopathies that have been experienced since adolescence; 50% of women suffer from obesity [10, 11].

For a more detailed understanding of the pathogenesis of the disease, clinical picture and prognosis for reproductive and somatic health, the phenotypes of PCOS were identified at the third consensus of the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) in Amsterdam in 2011. .

The diagnosis of PCOS in women should be confirmed at least with two of the three criteria: chronic anovulation, hyperandrogenemia (HA), the polycystic structure of ovary (20 or more follicles with at least one ovary volume over 10 cm³ in the absence of a dominant follicle and yellow body cyst). According to these features, there are four phenotypes of PCOS:

- I incomplete classic phenotype: HA (clinical and biochemical) and chronic anovulation (H-CA);
- II ovulatory form: HA and polycystic ovaries defined by ultrasound scanning (PCOm), but with ovulatory cycles (H-PCOm);
- III normoandrogenic form: chronic anovulation and non-HA polycystic ovaries (CA-PCOm);
- IV complete or classic phenotype: HA, chronic anovulation and polycystic ovaries (H-CA-PCOm) [12–15].

For a long time, medical care for PCOS patients aimed at restoring women's menstrual and fertile function. The purpose of the treatment was to restore the ovulatory menstrual cycle and fertility, eliminate the manifestations of androgen-dependent dermatopathy, and normalize body weight [16, 17]. However, according to some authors, the treatment approach for such women should be determined by the mechanisms of disease development. Today, priority is given to the complex treatment of women with PCOS,

taking into account their age, hormonal state, fertility, and there is a focus on the diagnostics and correction of metabolic disorders that impair the quality and life expectancy of patients [18–20].

In the last decade, modern science has been enriched by new data regarding the biological role of vitamin D, which suggested reviewing the importance of this vitamin in the body. There is increasing information on the role of vitamin D in the pathogenesis of not only the pathology of the bone system, but also obesity, DMII, dyslipidemia, and disorders of the reproductive system [21]. Currently, there is evidence that low levels of vitamin D are associated with obesity and, conversely, deficiency of this nutrient may predict the excessive adipose tissue. Some examinations have found an inverse correlation between 25(OH) D content and IR in women with PCOS. There is an opinion that high levels of this vitamin are associated with a high ovarian reserve, and hypovitaminosis D may be one of the factors that lead to premature ovarian depletion [22–26].

Vitamin D, which enters the body with food and is also produced by insolation, is biologically inert. In order to activate it, it is necessary to undergo two stages of hydroxylation. In the liver, it is converted to 25(OH) D, cholecalciferol, - its most common form. Level 25(OH) D indicates the content of this nutrient in the blood. Under the action of the enzyme 1- α -hydroxylase in the kidneys 25(OH) D produces its active metabolite (1,25 (OH) 2D₃, calcitriol,) through which the vitamin makes its clinical effect on the body. Thus, vitamin D can act both directly - through specific VDR receptors, and indirectly - stimulating the synthesis of steroid hormones (estrogen, progesterone, testosterone). These receptors are present in women's ovaries, endometrium, fallopian tubes, as well as in the decidual membrane [27, 28].

Nowadays there are strong proofs showing the role of vitamin D in the pathogenesis of PCOS. Some researchers have diagnosed the inverse correlation between anti-Mullerian hormone (AMH) levels in the blood and vitamin D in women with PCOS. Other scientists have proved that vitamin D deficiency causes a decrease in the content of sex-binding globulin (SHBG), which in

its turn leads to the development of HA, as well as to the increased synthesis of insulin and, as a result, hyperinsulinemia and insulin resistance (IR). A number of publications have demonstrated a correlation between vitamin D hypovitaminosis and testosterone and dehydroepiandrosterone sulfate (DHEA-S) levels. In vitro studies have shown that insufficiency of vitamin D in the body is accompanied by impaired follicle maturation, cyst formation, absence of yellow bodies, and stromal sclerosis [28, 30]. However, there is not enough data to fully understand the mechanisms through which vitamin D influences PCOS and how appropriate it is to use this nutrient in complex therapy of PCOS.

The Goal of the Study

To explore the role of vitamin D for effective treatment of women with PCOS depending on the phenotype.

Materials and Methods

To achieve this goal, we examined 80 women, aged 18-35 years, with PCOS and underlying overweight and obesity (experimental group) and 20 women of same age in good physical and gynaecological health (control group). The studies were performed at the Department of Endocrinology, LNMU by Danylo Halytskyi and Intersono Clinic in Lviv (Ukraine). Diagnostics of PCOS was performed according to Rotterdam requirements. Exceptional cases were women with severe endocrine and somatic pathology and conditions that were contraindications to COCs.

All women underwent a general medical examination, having measured their anthropometric parameters, body mass index (BMI) and clinical manifestations of HA (hirsutism score (HS) by Ferriman-Gallwey scale). Pelvic ultrasound was performed by Siemens ultrasound machine (Germany) and laboratory tests were taken at the Intersono Medical Center. The levels of LH, FSH, progesterone (Ps), estradiol (Es), androstenedione (A), dehydroepiandrosterone sulfate (DHEA-s), 17-hydroxyprogesterone

(17-OHP), anti-Mullerian hormone (AMH), insulin, leptin were measured with ECLIA method - electrochemiluminescent immunoassay (using automatic analyzers and reagents of Roche Diagnostics (Germany)) and enzyme-immunoassay (using standard kits of Immunotech (Czech Republic)) on 3rd-5th days of the menstrual cycle. The index of free testosterone (FAI) was calculated by the formula: $FAI = TT / SHBG \times 100$. The content of 25(OH)D in serum was measured by chemiluminescent immunoassay using LIAISON analyzer and LIAISON 25OH TOTAL kits (DiaSorin Inc., USA). Vitamin D deficiency was diagnosed at the level of 25(OH) D at <20 ng/mL, deficiency at concentration of 25(OH) D at 20-30 ng/mL, adequate levels - > 30 ng/mL. The insulin resistance index (IR) was calculated by the NOMA-IR formula: $\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mcU/mL)} / 22.5$.

All women were divided into four phenotype groups, according to the consensus of the PCOS Workshop Group in Amsterdam (2011). Group 1 included 20 women with HA and polycystic ovaries (ovulating); Group 2: 19 women with HA and chronic anovulation (no multifollicular ovaries); Group 3: 23 women with polycystic ovaries and anovulation (no signs of HA); Group 4: 18 women with HA, chronic anovulation and polycystic ovaries (complete syndrome). In addition, all patients in each group were further divided into two subgroups, depending on the BMI. The 1st subgroup included overweight women (BMI of 25.29.9 kg/m²), and the 2nd group consisted of obese patients (BMI > 30 kg/m²). We have been thoroughly studying the medical history of patients and their lifestyles, evaluating objective data and complaints. The treatment approach was chosen according to the identified PCOS phenotype and metabolic disorders. Half of the patients of each phenotype group, at their request, were additionally prescribed a vitamin D dose of 2000-4000 IU (depending on the background level of the nutrient). The effectiveness of the therapy was evaluated after six months of treatment.

Statistical processing of the obtained data was performed using standard methods of descriptive and categorical statistics and the Statistica 8.0 Certified Software Package (Statsoft

Inc., USA). Quantitative indices were presented as mean $\pm$ standard deviation. Quantitative indices in groups were compared using the Mann-Whitney U-test. The correlation between quantitative indicators was studied by Spearman's rank correlation method. The difference was considered significant in the case of $P < 0.05$. Examination of patients was carried out in accordance with the requirements of the Declaration of Helsinki (2004).

Results of the Study and Discussion

Having analyzed the hormonal state in women with PCOS in different phenotypic groups, we found in their blood a significant increase in the LH levels, a decrease in the concentration of FSH and insufficient content of Es and Ps, especially in patients with chronic anovulation ($P < 0.01$); a tendency for an increase in FAI and androgens, especially in phenotypic groups with present HA; an increase in the AMH level, in particular in women with PCOS (Table 1).

Analysis of leptin content in women with PCOS defined the presence of hyperleptinemia in all examined patients. The concentration of

leptin in their blood increased proportionally to the increase in BMI.

The dynamics of leptin concentration was accompanied by similar changes in insulin content and NOMA-IR scores, in particular, higher insulinemia and NOMA-IR were diagnosed in the PCOS group of women with verified obesity, which increased with the degree of obesity; this allows to consider the excess body mass as an adverse factor provoking the development of hyperinsulinemia and progression of IR in this nosology (Table 2).

When analyzing the levels of 25(OH) D in the serum of the examined women, we have noted a tendency for a decrease in average vitamin D concentrations in patients of all PCOS phenotypic groups compared to control groups. In most patients, vitamin D content did not correspond to its normal level (21.8% and 36.1% in groups 1 and 2, respectively). Both insufficiency and deficiency of cholecalciferol varied significantly among PCOS patients as opposed to healthy women. Their cholecalciferol deficiency increased with the development of obesity (Table 3).

Analysis of metabolic examinations has shown that IR was combined with

Table 1: Hormonal state in women with PCOS depending on phenotype.

Index	Control group (n=20)	Experimental group (PCOS) (n=80)			
		Group 1 (n=20)	Group 2 (n=19)	Group 3 (n=23)	Group 4 (n=18)
LH, mIU/mL	7,3 $\pm$ 3,5	13,3 $\pm$ 3,5*	15,6 $\pm$ 2,7*	16,3 $\pm$ 2,5*	18,3 $\pm$ 3,2*
FSH, mIU/L	5,4 $\pm$ 2,1	5,1 $\pm$ 1,1	4,3 $\pm$ 0,28*	4,2 $\pm$ 0,32*	3,8* $\pm$ 0,41*
Es, pmol/L	163,0 $\pm$ 11,8	157,1 $\pm$ 11,3	97,1 $\pm$ 14,2*	78,1 $\pm$ 8,6*	77,4 $\pm$ 12,3*
Ps, nmol/L	1,43 $\pm$ 1,22	1,12 $\pm$ 0,13	0,58 $\pm$ 0,14*	0,61 $\pm$ 0,13*	0,62 $\pm$ 0,13*
SHBG, nmol/L	94,6 $\pm$ 10,2	35,4 $\pm$ 8,3*	43,4 $\pm$ 7,1*	85,4 $\pm$ 9,3	35,4 $\pm$ 8,3*
fT, nmol/L	1,6 $\pm$ 0,1	4,6 $\pm$ 0,4*	6,6 $\pm$ 0,2*	1,8 $\pm$ 0,4	4,6 $\pm$ 0,4*
FAI, nmol/L	60 $\pm$ 20,1	13 $\pm$ 2,1*	12 $\pm$ 0,7*	30 $\pm$ 10,1	10 $\pm$ 1,8*
17-OHP, ng/mL	0,72 $\pm$ 0,11	0,71 $\pm$ 0,13	0,81 $\pm$ 0,77	0,68 $\pm$ 0,77	0,71 $\pm$ 0,77
A, nmol/L	3,8 $\pm$ 0,22	15,6 $\pm$ 1,14*	17,8 $\pm$ 1,22*	4,6 $\pm$ 0,33	16,8 $\pm$ 1,44*
DHEA, mcg/mL	176,4 $\pm$ 11,7	484,6 $\pm$ 26,3*	574,6 $\pm$ 28,2*	184,6 $\pm$ 14,3	684,6 $\pm$ 28,1*
AMH, ng/mL	3,8 $\pm$ 0,3	4,1 $\pm$ 1,2	6,1 $\pm$ 0,3*	10,1 $\pm$ 1,1*	11,1 $\pm$ 0,8*
HS (hirsutism score)	6 $\pm$ 1,2	13 $\pm$ 0,4*	13 $\pm$ 1,2*	8 $\pm$ 0,8	14 $\pm$ 1,3*

Note. * – the significance compared to the control group, $P < 0.01$.

Table 2: Indices of leptin, insulin, and HOMA-IR in the serum of women with PCOS depending on the phenotype.

Index	Control group (n=20)	Experimental group (PCOS) (n=80)							
		I n = 20		II n = 19		III n = 23		IV n = 18	
		1	2	1	2	1	2	1	2
HOMA-IR	1,77±0,11	2,71±0,1	4,32±1,1*	2,43±1,23	4,43±1,23*	2,52±2,45	3,52±2,45*	3,12±3,44	6,12±3,44*
Insulin mIU/mL	8±2,32	11±0,21	22±0,21*	14±2,41	24±2,41*	13±3,21	28±3,21*	16±3,34	34±3,34*
Leptin, ng/mL	65±11,2	74±6,12	86±6,12*	77±5,1	82±9,1	74±6,2	83±10,4*	76±7,2	88±13,2*

Note. * – the significance compared to the control group, $p < 0,01$.

Table 3: Vitamin D level in the serum of women with PCOS.

Group	Deficiency %		Insufficiency %		Optimal level %	
Group 1 (women with PCOS)	36,3		48,4		15,3	
	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)
	11,2	25,1	14,7	33,7	9,2	6,1
Group 2 (healthy women)	13,5		31,9		54,6	
	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)	Subgroup 1 (BMI 2529,9)	Subgroup 2 (BMI >30)
	6,2	7,3	14,3	17,6	22,5	32,1

hyperleptinemia, compensatory HI and vitamin D hypovitaminosis in women with obesity in all clinical PCOS phenotypes, which is also similar to the results of other scientists [31,32,33,34].

In the correlation analysis of vitamin D content and hormonal indices in women with PCOS, we have established an inverse correlation between levels 25(OH) D and AMH, especially in patients with polycystic ovaries; found a positive correlation between FAI and 25(OH) D levels in the blood plasma, as well as a negative correlation between vitamin D levels and hirsutism score, especially in the subgroup of patients experiencing HA, which also coincides with the results of studies of other researchers (Table 4) [35,36].

Table 2a and Table 3a: M – mean of index in the group; δ – mean squared deviation; r (X,Y)

— correlation coefficient; p — the probability of a correlation coefficient error.

Therefore, a decrease in vitamin D is associated with an increase in BMI, an increase in free testosterone level, luteal phase defect (a decrease in progesterone), ovulatory dysfunction and IR progression. Our study confirms the role of vitamin D in the pathogenesis of PCOS, according to which hypovitaminosis D can be considered as one of the factors causing impaired reproductive function in women, which has been proven by other authors [37,38].

The subtle pathogenetic mechanisms underlying the correlation between cholecalciferol deficiency and insulin resistance and HI are still not fully understood, in particular, it is unclear whether vitamin D

Table 4: Correlation of anthropometric and hormonal indices with vitamin D content in women with PCOS.

Index	M	Δ	r (X,Y)	p
ng/mL	110,4	34,3		
BMI, kg/m ²	33,11	5,16	0,751	0,001
WC (Waist circumference), cm	107,23	10,60	0,572	0,001
DHEA-s, ng/L	3,27	1,22	0,241	0,322
Insulin, mIU/mL	29,41	10,24	0,762	0,001
AMH, ng/mL	6,64	2,5	0,116	0,124
LH, mIU/mL	13,12	4,11	0,212	0,220
FSH, mIU/mL	6,87	3,64	-0,171	0,325
Testosterone, total, ng/mL	2,72	0,51	0,221	0,483
Estradiol, pg/mL	0,64	0,24	0,251	0,532
Progesterone, ng/mL	4,73	1,53	0,117	0,002
SHBG, pg/mL	76,91	46,16	0,074	0,630
Androstendione, ng/mL	12,68	6,23	0,010	0,953
Testosterone, free, pg/ml	7,41	1,52	0,078	0,037
HS (hirsutism score)	11,24	2,45	0,582	0,044

deficiency is a result of obesity or whether obesity is a consequence of vitamin D insufficiency. On the other hand, obesity can initiate a decrease in circulating 25(OH) D levels due to the absorption of cholecalciferol by adipose tissue. A decrease in vitamin D can be considered a trigger

forming hormonal imbalances in women with hyperandrogenic status.

Table 5 presents the changes in the hormonal state of women with PCOS after treatment with vitamin D.

Table 5: Hormonal state of women with PCOS after treatment with vitamin D.

Index	Control group (n=20)	Experimental group (PCOS) (n=80)			
		Group 1 (n=20)	Group 2 (n=19)	Group 3 (n=23)	Group 4 (n=18)
LH, mIU/mL	8,1±2,5	9,3±3,2*	10,6±2,5*	8,3±2,6	11,3±2,2*
FSH, mIU/L	5,4±2,1	5,3±1,1	6,3±0,28	4,9±0,32	4,8±0,41
Es, pmol/L	173,0±12,8	165,1±10,3	117,1±11,2*	98,1±8,2*	97,4±10,3*
Ps, nmol/L	1,53 ±1,22	1,32±0,13	1,28±0,13*	0,99±0,12*	1,12±0,11*
SHBG, nmol/L	94,6±10,2	55,4±8,3*	53,4±7,1*	85,4±9,3	55,4±8,3*
TT, nmol/L	1,6±0,1	2,6±0,4*	4,2±0,2*	1,7±0,4	3,6±0,4*
FAI, nmol/L	58 ±18,1	33 ±12,1*	22 ±6,7*	42 ±10,1	10 ±1,8*
A, nmol/L	3,8±0,22	15,6±1,14*	17,8±1,22*	4,6±0,33	16,8±1,44*
DHEA, mcg/mL	176,4±11,7	484,6±26,3*	574,6±28,2*	184,6±14,3	684,6±28,1*
AMH, ng/mL	3,8±0,3	4,1±1,2	6,1±0,3*	10,1±1,1*	11,1±0,8*
HS (hirsutism score)	6±1,2	13±0,4*	13±1,2*	8±0,8	14±1,3*

Note. * – the significance compared to the control group, p<0,01.

Results of treatment with vitamin D demonstrated a decrease in the free testosterone index, a significant decrease in hirsutism score, an increase in blood progesterone concentration (in the luteal phase), and an increase in the number of ovulations in women with PCOS.

The findings of the study indicate a deficiency of vitamin D in patients with PCOS, correspond to global science data and show the need for more research to examine the role of cholecalciferol in ovarian dysfunction. Today, there is much evidence stating that a violation of the D-vitamin content in a woman's body leads to disruption of the ovarian menstrual cycle, takes part in the development of PCOS and premature ovarian depletion. This recommends defining the level of 25(OH) D in blood plasma for the timely detection and correction of hypovitaminosis in women with the specified pathology.

Conclusions

Women with PCOS (regardless of specific phenotype) are diagnosed with a decrease in (25 (OH) D), insufficiency of which is exacerbated as obesity progresses. The study found out the correlation between vitamin D content in the blood and the degree of obesity, HI and IR in women with PCOS. Prescription of vitamin D in the complex treatment of women with PCOS leads to normalization of hormonal state, reduction of HA signs, normalization of the menstrual cycle, and restored ovulation.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Serum Testosterone and Metabolic Syndrome in Old-Aged Males: Preliminary Findings of the AHAP Cohort Study

Aylar Khalilipour¹, Nazila Malekian¹, Haleh Esmaili², Seyed Reza Hosseini², Mohammadali Bayani³, Ali Bijani², Fatemeh Izadpanah¹, Reza Ghadimi^{2*}

¹ Student Research Committee, Babol University of Medical Sciences, Babol, Iran

² Social Determinants of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

³ Department of Internal Medicine, Babol University of Medical Sciences, Babol, Iran

*Correspondence to: Reza Ghadimi, Social Medicine, Babol University of Medical Sciences, 47176-47745, Ganjafrroz Street, Babol, Mazandaran, Iran. E-mail: rezaghadimi@yahoo.com. Phone: +98 11 321190557; Fax: +98 11 32190557

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Abstract

Introduction: Metabolic syndrome (MetS) is one of the critical health concerns. The relationship between low total testosterone and MetS was reported in some studies. However, the association independent of other related components is not fully known. This study was performed to evaluate the prevalence rates of MetS among older men with and without hypogonadism and the related association. **Material and Methods:** This cross-sectional study was conducted on 800 male subjects aged 60 years and older. Anthropometric indices, blood pressure, total serum testosterone, and serum lipid profile were determined. The Iranian National Committee of Obesity criteria were used to define metabolic syndrome. **Results:** The mean total testosterone in the MetS group was significantly lower compared to the non-MetS group. Total testosterone showed significant inverse associations with triglycerides and waist circumference. The crude odds ratio for MetS between the low and high total testosterone groups was 1.43, which remained statistically significant after adjustments, while total testosterone levels showed no significant association with MetS after adjusting by BMI. **Conclusion:** Testosterone is negatively and independently associated with MetS regarding age, smoking status and alcohol intake in Iranian men. Therefore, the early diagnosis and treatment of hypogonadism should be considered in MetS prevention.

Keywords: Metabolic syndrome, testosterone, aged.

Introduction

Metabolic syndrome (MetS), as a cluster of medical conditions, is one of the most important health concerns and includes obesity, high blood pressure, high fasting plasma glucose and serum triglyceride, and low high-density lipoprotein (HDL) levels [1]. In the last decade, the prevalence of MetS is increasing in the world, varying between races and populations [2]. The prevalence of MetS in the United States of America, Asian Pacific, and East and Southeast Asia is increased, reaching a value of around 25% [3, 4]. Iran, as an Asian country, is not an exception. The

results of two meta-analysis studies have shown a higher prevalence of MetS in males (about 26%) [5, 6]. Although only a partial mechanism of the MetS pathway is known, it is clear that food habits, sedentary lifestyle, stress, and other environmental factors have crucial roles [7].

Furthermore, the inverse relationship between total testosterone (TT) and sex hormone-binding globulin (SHBG) levels with MetS has been illustrated [8]. Therefore, low sex hormone levels (total testosterone and SHBG) may be a risk factor for MetS, just like increased insulin resistance and adipose tissue [9, 10]. Hence, a low testosterone level is one of the possible



risk factors for MetS. It is not clear whether the relationship of low TT to MetS is independent of BMI and waist circumference or not [11, 12]. Besides, the relationship between testosterone components and MetS is known clearly. Although the free testosterone level has an inconsistent relationship with some MetS components, SHBG has demonstrated a constant inverse relationship [13]. Therefore, in this study, we measured serum total testosterone (TT) and evaluated several metabolic factors, such as waist circumference, diabetes, dyslipidemia, hypertension, and hyperglycemia, among healthy older men to clarify this association. This large-scale cross-sectional study aims to determine and compare the prevalence of MetS among men with and without hypogonadism in Amirkola, Mazandaran province, North of Iran.

Material and Methods

Study design and patients

This cross-sectional study was a part of the Amikola Health and Aging Project (AHAP) [14] and was approved by the Medical Research Ethics Committee of Babol University of Medical Sciences. This study was conducted on 800 healthy males aged 60 years old and older. Subjects were excluded if they had malignancies or acute illnesses such as liver cirrhosis, diabetes mellitus type I and II, infection, and inflammation. Also, men who were under testosterone replacement therapy or taking anti-androgen and steroidal agents were excluded. All participants signed an informed consent form.

Laboratory, anthropometric and clinical data collection

Weight (to the nearest 0.1 kg) and height (to the nearest 0.1 cm) were measured following standard procedures; waist and hip circumference was also measured to the nearest 0.5 cm in all participants with a non-elastic tape. Body mass index (BMI) was calculated by dividing the weight (kilograms) by the square of height

(meters). Blood pressure was measured using standardized sphygmomanometers by a trained nurse after a 10-15 min rest in a sitting position. Venous blood was collected in the morning in a fasting state to detect total testosterone, fasting blood sugar (FBS), total cholesterol (T-Chol), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG). Total testosterone levels were measured using a DiaMetra kit made in Germany by the ELISA method (ng/ml). The subjects with a history of androgenic drug intake during the last month were excluded from the study.

BMI 18-25 kg/m² were defined as normal, 25 -30 kg/m² and ≥ 30 kg/m² were defined as overweight and obese, respectively. To define MetS, we used the Iranian National Committee of Obesity (INCO) criteria [15]. According to INCO, metabolic syndrome involves having at least three of the following symptoms: waist circumference >95 cm, triglyceride level >150 mg/dl (1.7 mmol/L), HDL-C <40 mg/dl (1.03 mmol/L), blood pressure $> 130/85$ mmHg and fasting plasma glucose > 100 mg/dl (5.6 mmol/L); or patients were under medical treatment [15]. According to the Clinical Practice Guidelines of the Endocrine Society for androgen deficiency syndromes in adult men, hypogonadism was defined as total testosterone < 3.0 ng/mL [16, 17]. Therefore, according to total testosterone, subjects were divided into two groups. Group (1): TT ≥ 3 as a reference group and group (2): TT < 3 as the hypogonadal group.

Statistical analysis

The values were expressed as mean $\pm$ standard deviation as a percentage for descriptive statistics. Mean differences of total testosterone between the MetS group and reference group were assessed using the independent sample t-test. Different MetS prevalence, according to each group, was calculated using the chi-square test. The relationship between total testosterone and MetS were assessed with logistic regression analysis after adjusting for age and BMI. Statistical significance was set at $p < 0.05$. Statistical analyses were performed with SPSS version 18.0.

Results

The mean age of the subjects was 69.7 ± 7.5 years, and the prevalence of MetS, according to the INCO criteria, was 64.9%. The average serum total testosterone was 4.78 ± 4.1 ng/ml. According to the BMI, 44.3% of subjects were overweight and 15.2% were obese. About 34.3% were current smokers, and 4.4% were alcoholics.

Table 1 shows the general characteristics of the study subjects. The mean total testosterone of the MetS group was significantly lower than that of the non-MetS group (4.5 ± 4.1 ng/ml vs. 5.1 ± 4.08 ng/ml; $p=0.042$). The mean values of MetS components in hypogonadal men and

reference groups are presented in Table 1. The average waist circumference ($p=0.03$), hip circumference ($p=0.016$), and BMI ($p=0.005$) were significantly different among the two groups. However, there was no significant difference in age, LDL-C, HDL-C, TG, total cholesterol, FBS, and systolic/diastolic blood pressure (BP).

The risk of metabolic syndrome was statistically significant in the crude model but also after adjustment for age, smoking status, and alcohol intake (model 1). However, TT showed no significant association with MetS after adjustment for BMI (model 2) (Table 2). Also, the odds ratio of the hypogonadal group for MetS components were assessed (Table 3). Total testosterone

Table 1: General characteristics of the subjects and mean values of MetS components in hypogonadal and reference groups.

Characteristics	Mean(SD)			p-value
	Total	TT<3ng/ml (n:263)	TT≥3 ng/ml (n:527)	
Age (year)	69.74 (7.59)	70.23 (8.26)	69.49 (7.23)	0.198
BMI (kg/m ²)	26.06 (4.00)	26.63 (4.13)	25.78 (3.91)	0.005*
Waist circumference (cm)	95.06 (10.38)	96.19 (10.91)	94.50 (10.07)	0.031*
Hip circumference (cm)	99.86 (7.71)	100.8 (8.17)	99.39 (7.44)	0.016*
Systolic blood pressure (mmHg)	142.73(21.95)	142.21 (20.95)	142.99 (22.44)	0.636
Diastolic blood pressure (mmHg)	81.14 (12.05)	81.29 (11.91)	81.07 (12.14)	0.812
Fasting blood sugar (mg/dl)	112.97 (40.09)	112.61 (38.12)	113.14 (41.07)	0.861
Total-cholesterol (mg/dl)	188.07 (38.46)	188.76 (44.44)	187.72 (35.17)	0.723
TG (mg/dl)	148.83 (79.14)	153.38 (82.93)	146.56 (77.16)	0.254
HDL-C (mg/dl)	38.41 (4.06)	38.35 (4.34)	38.43 (3.92)	0.792
LDL-C (mg/dl)	122.63 (39.90)	123.02 (44.39)	122.43 (37.51)	0.846
Total testosterone (ng/ml)	4.77 (4.11)			

Note: BMI: Body Mass Index; FBS: Fasting Blood Sugar; BP, Blood Pressure; * p-value less than 0.05 was considered significant.

Table 2: Odds ratio of metabolic syndrome according to the TT level.

Group	Crude OR (CI)	Model1 OR (CI)	Model2 OR (CI)
TT≥3 (ng/ml)	1	1	1
TT <3 (ng/ml)	1.43(1.047-1.977)*	1.51(1.089-2.092)*	1.245(0.860-1.802)

Note: Model 1: adjusted for age, smoking status, alcohol intake;
Model 2: adjusted for all covariates in model 2 and BMI.

Table 3: Odds ratios of metabolic syndrome components according to the TT level.

Group	BP >130/85 (mmHg) OR (CI)	FBS >100 (mg/dl) OR (CI)	WC ≥94 (cm) OR (CI)	TG >150 (mg/dl) OR (CI)	HDL <40 (mg/dl) OR (CI)
TT ≥3 (ng/ml)	1	1	1	1	1
TT <3 (ng/ml)	0.975 (0.677–1.404)	1.067 (0.793–1.437)	1.481 (1.091–2.009)*	1.354 (1.006–1.824)*	1.078 (0.77–1.509)

Note: Model 1: adjusted for age, smoking status, alcohol intake;
Model 2: adjusted for all covariates in model 2 and BMI.

showed significant strong associations with TG (OR: 1.354, $p=0.04$) and WC >94 (OR: 1.481, $p=0.01$). Therefore, subjects with low TT levels had a risk of higher TG and WC values compared with the normal group.

Discussion

Different cutoff values for total testosterone were used to define hypogonadism. In our study, the cutoff level of 3.0 ng/ml for total testosterone was used to define hypogonadism as recommended by the Clinical Practice Guidelines of the Endocrine Society [18, 19]. This level of testosterone was considered enough to improve certain parameters of sexual function in men that suffer from hypogonadism [20]. In this regard, since hypogonadism is not a specific MetS symptom, it is challenging to diagnose and treat. Therefore, it is important to combine the MetS symptoms of androgen deficiency with low testosterone levels to establish the diagnosis of clinical hypogonadism [20]. In the present cross-sectional study, we showed a clear significant association of total testosterone levels with MetS independent of age and BMI in generally healthy Iranian men. Meanwhile, total testosterone was associated with only two components of MetS, e.g., TG and waist circumference. Similarly, a study in Taiwan concluded that waist circumference and total testosterone levels are strongly associated [21] while other studies depicted that a low level of sex hormones have been associated with many risk factors for MetS such as central obesity, insulin resistance, diabetes, and unfavorable lipid profile, which are prominent in men with a normal BMI and the negative relationship of lower

levels of sex hormone and MetS is consistent with other studies [11, 22–25]. Additionally, in the present study, the mean value of BMI in the hypogonadal group was significantly greater than the reference group. TT level was lower in men with MetS compared with men without MetS and both type 2 diabetes and MetS independently predicted low testosterone level for age and BMI. The testosterone level decreased significantly along with an increase in the number of MetS components, showing a significant correlation even after adjustment for age, BMI, and waist circumference [26, 27]. This association between BMI and low testosterone was reported in several studies [23, 28, 29], and low serum testosterone levels could also be considered as one of the many adverse consequences of obesity [30, 31].

On the other hand, through the conversion of testosterone to estradiol by the aromatase enzyme in obese subjects, obesity is considered as a factor that is associated with subnormal testosterone levels. However, we did not measure estradiol and were unable to assess the possible role of aromatization on these outcomes. However, a study that included Japanese men showed that a decrease in androgen production and low or inappropriately normal levels of luteinizing hormone in obese middle-aged men moderately led to a decrease in testosterone production [32]. Low sex hormones and MetS produce a vicious circle with synergic characteristics through bidirectional effects in which low sex hormone levels lead to the development of MetS, which, in turn, is associated with a further decline in sex hormone levels [33, 34]. Therefore, it seems that total testosterone and SHBG have a protective role against MetS as high testosterone and SHBG were associated with a decrease in

the MetS risk, independent of body composition [34]. Another study on middle-aged Japanese men also reported that serum testosterone level was significantly related to the MetS associated conditions, included obesity and hypertension. Compared to the highest tertile, subjects in the lowest tertile of testosterone were approximately four times more likely to have diabetes [28]. A linear increase in serum TGs levels was found in men with low TT and SHBG, which was inversely and independently associated with TG, and positively and independently associated with HDL [35]. Moreover, men with HDL < 0.90 and TG > 1.8 had significantly lower levels of TT and SHBG [35]. In our study, hypogonadism was associated significantly with hypertriglyceridemia, but the mean values of TG showed no significant difference between the two groups. We suggest that an association between low serum TT level and hyperglycemia can cause metabolic changes, rather than the direct effect. To the best of our knowledge, many studies on testosterone and MetS have been performed in Western countries, whereas relatively few studies for Asians have been reported despite the different definitions of MetS. Our study analyzed a large sample from the Iranian population, which can support data on the effects of sex hormones on MetS in Iranian men. However, the present study contains some limitations. First, it was a cross-sectional study, which explains associations but not causality. Second, as our data were from the general Iranian population, it is not enough to expand to other ethnic/racial groups. Third, we used radioimmunoassay instead of mass spectrometry for total testosterone determination. Data derived using immunoassay is technically simple, rapid, and relatively inexpensive at the cost of poor accuracy and sensitivity. However, immunoassay of total testosterone is a widely accepted method used in large studies since the measurement of total testosterone by mass spectrometry is impractical. Fourth, free testosterone and sex hormone-binding globulin analyses and levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were not determined; thus, we were not able to classify the type of hypogonadism, whether it is primary or secondary.

Conclusions

In conclusion, testosterone is negatively associated with MetS independently and after adjustment for age, smoking status, and alcohol intake among Iranian men. Among MetS components, TG and WC were strongly associated with testosterone levels among elderly males regardless of obesity. Further studies, including clinical trials, are needed to confirm our findings.

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Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Levels of pro-inflammatory cytokines (IL1 β , IL18) and C-reactive protein in patients with diabetic retinopathy

Marta Horecha¹, Lyubov Lapovets^{1*}, Nataliia Lapovets², Viorika Akimova¹, Natalija Bojkiv¹, Oksana Tsymbala¹, Sergii Tkachuk¹, Natalia Lisnianska³

¹ Department of Clinical Laboratory Diagnostics, Danylo Halytskyi Lviv National Medical University, Lviv, Ukraine

² Research Institute of Epidemiology and Hygiene, Danylo Halytskyi Lviv National Medical University, Lviv, Ukraine

³ Department of Pathological Physiology, Bukovinian State Medical University, Chernivtsi, Ukraine

*Correspondence to: Lyubov Lapovets, Department of Clinical Laboratory Diagnostics, Danylo Halytskyi Lviv National Medical University, Pekarska Street, 69, Lviv, 79010, Ukraine. E-mail: lapovets@ukr.net Phone: +380936508311

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Abstract

Background and Aims: Diabetic retinopathy is a major complication of diabetes mellitus (DM), which remains a leading cause of visual loss in working-age populations. This study aimed to investigate serum levels of IL1 β , IL18, and C-reactive protein (CRP) in patients with diabetic retinopathy. **Material and Methods:** A total of 70 insulin-dependent patients and 60 non-insulin dependent patients with diabetic retinopathy were enrolled in the study. Interleukin levels were assessed using “Vector-Best”, Ukraine reagent kits on a STAT FAX 303 plus analyzer. The level of CRP was determined using a CRPLX set of reagents “Roche Diagnostics”, Germany on a COBAS INTEGRA 400 plus automatic analyzer. **Results:** In insulin-dependent patients there were no statistically significant differences in IL1 β and IL18 levels in the control group. Serum level of IL1 β in non-insulin dependent patients was 43.4% higher than in the control group, and 50.0% higher than in insulin-dependent patients. Serum level of IL18 in non-insulin dependent patients was 12.3% higher than the control group and 11.4% higher than the insulin-dependent patients. Also, we have found that the level of CRP in insulin-dependent patients increased by 24.1% in the control group. In non-insulin dependent patients CRP level did not change significantly in the control group. **Conclusions:** The obtained results suggest that insulin-dependent patients with diabetic retinopathy demonstrate predominance of acute inflammation, which is confirmed by acute phase marker increasing, and normal levels of pro-inflammatory cytokines in blood serum. Patients with diabetic retinopathy who do not use insulin were found to have increased serum levels of IL1 β and IL18 and normal level of CRP, which suggests the predominance of pro-inflammatory processes (mobilization and activation of cells typically involved in inflammatory process).

Keywords: Diabetic retinopathy, Inflammation, Type 2 diabetes mellitus.

Background and Aims

Diabetes mellitus (DM) is a multifactorial metabolic disorder, characterized by chronic hyperglycemia leading to significant physiological, biochemical, and histological changes in the affected organisms [13]. For about half a century, scientists around the world have been thoroughly engaged in the problem of chronic hyperglycemia, but the causes of its occurrence have not been fully

established yet. The most relevant modern theory is the autoimmune inflammation in diabetes pathogenesis [4, 5]. Pathological effects of hyperglycemia are seen in the form of diabetic complications which are equally health-devastating in nature as the disorder of diabetes itself [6, 7]. Despite prolonged studies of the mechanisms behind the development of diabetic retinopathy, as well as thorough investigation into the main stages of its pathogenesis, many aspects of this condition, including the importance



of imbalance in pro- and anti-inflammatory cytokines, remain the subject of ongoing scientific debate.

There are data that confirmed the role of different cytokine classes in diabetic retinopathy [8, 9] pathogenesis. Recent studies have shown that both interleukin 1 β (IL1 β) and tumor necrosis factor alpha (TNF α) are capable of slowing down retinal endothelial migration and capillary morphogenesis [10]. Other scientific evidence suggests that these interleukins may be playing an important role in disrupting the integrity of the gamma-retinal barrier, the occurrence of retinal leukostasis, and activation of apoptosis in proliferative diabetic retinopathy. Hyperglycemia is viewed as a trigger and retinal endothelium as a source of hyperexpression of IL1 β , maintaining its own increased expression by self-stimulation of endothelial cells and macroglia [11].

IL18 is a member of the family of pro-inflammatory cytokines that stimulate the production of IFN γ , TNF α , IL1, IL2, adhesion molecules, and apoptosis factors as well as increase the proliferative activity of T-lymphocytes and activate NK cells. IL18 is involved in cellular and humoral immunity, as well as in innate and acquired immune responses [12]. The above effects of this cytokine allow viewing it as one of the key factors for anti-infective and anti-tumor protection of the body. In addition, there is evidence that in some cases IL18 may act as a pathogenetic factor in some diseases accompanied by acute and chronic inflammation.

C-reactive protein (CRP) is a multifunctional acute phase protein involved in protecting the body from pathogens in a setting of autoimmune and inflammatory processes and it is quite a well-known fact that inflammation is one of the most crucial determining factors in the progression of various diabetic complications [6]. In autoimmune processes, the involvement of CRP is actuated through binding to the ligand, which provides the destruction of autoimmune determinants with loss of antigenic properties. Accordingly, the complement system is activated by stimulating phagocytosis and by eliminating toxic products. CRP is absorbed by neutrophils, where immunoactive peptides are produced during proteolysis in phagosomes; these peptides

are capable of modulating various neutrophilic and macrophageal functions. There are data about local CRP synthesis by activated macrophages and endothelial cells [13].

Due to peculiar characteristics of cytokinemia in type 2 diabetes (T2D), as the disease progresses in the patients, there is a growing number of cellular structures with high cytokine production. The research into the role of cytokinemia and the imbalance between pro- and anti-inflammatory cytokines in severe complications of T2D, such as diabetic retinopathy, will provide additional laboratory criteria for assessing the severity and the origins of this disease.

Therefore, our aim of study was to investigate serum levels of IL1 β , IL18, and CRP in patients with diabetic retinopathy.

Material and Methods

The study included a clinical and laboratory examination performed in 130 patients with diabetic retinopathy (70 insulin-dependent patients constituted Group 1, and 60 non-insulin dependent patients constituted Group 2). The average age of patients ranged from 20 to 55 years. The results of laboratory findings were compared with the control group, which included 30 healthy individuals.

Inclusion criterion: All patients suffering from diabetes, irrespective of the duration of the disease, were included in the study. The pattern of diabetic retinopathy was decided on the basis of ophthalmoscopic findings and fundus photograph.

Exclusion criterion: Patients with media haze obscuring visualization of fundus, and pregnant women were excluded from the study.

Venous blood samples were obtained from all patients, 5 ml per sample. Blood was collected in plastic tubes (BD vacutainer type) with double coagulation activator. All study subjects had their serum levels of IL1 β and IL18 assayed. Interleukin levels were assessed using “VECTOR-BEST Ukraine” reagent kits on a STAT FAX 303 plus analyzer.

The levels of CRP were determined using a CRPLX set of reagents by Roche Diagnostics on a COBAS INTEGRA 400 plus automatic analyzer.

Statistical processing of digital data was carried out using the software Excel (Microsoft, USA) and STATISTICA 8.0 (Statsoft, USA). The pattern of the distribution of the studied variables was assessed using the Shapiro-Wilk's criterion. Quantitative characteristics were represented as $M \pm m$ (arithmetic mean $\pm$ standard error of arithmetic mean). Under the normal distribution, the validity of the differences was estimated using the Student's t-criterion; under the distribution different from normal, Mann-Whitney's non-parametric U-criterion was used. Differences were considered statistically significant at $p < 0.05$.

Ethical Approval

The ethical principles contained in the Declaration of Human Rights adopted in Helsinki, in 1975, and revised in 2008, were fully respected in our study. The subjects enrolled voluntarily, participated in this study, and completed and signed a written informed consent. Protocol of study was approved by the local ethics committee of Danylo Halytskyi Lviv National Medical University.

Results

In the patients of Group 1, there were no statistically significant differences in IL1 β

and IL18 levels vs control group (Table 1). Serum levels of IL1 β in patients of Group 2 were 43.4% higher than those in the control group, and 50.0% higher than those in Group 1. Serum levels of IL18 in Group 2 were 12.3% higher than in the control group and 11.4% higher vs Group 1.

In order to assess the level of systemic inflammation, we tested the level of CRP, a common marker of acute inflammation; the increasing CRP is a known factor in progression of diabetic retinopathy. We have found that the levels of CRP in patients with diabetic retinopathy increased in insulin-dependent patients (Group 1) – by 24.1% compared with the control group (Table 1). In group 2 CRP levels were within normal range.

When calculating the CRP to IL1 β ratio, we found a 1.3-fold increase (2.4 ± 0.04 and 1.8 ± 0.05 , respectively) ($p < 0.05$) in patients of Group 1 vs control group. In patients of Group 2, there was a 1.4-fold decrease in the CRP to IL1 β ratio vs control group (1.3 ± 0.05 and 1.8 ± 0.05 , respectively) ($p < 0.05$) and a 1.8-fold decrease relative to patients of Group 1 (1.3 ± 0.05 and 2.4 ± 0.04 , respectively) ($p < 0.05$).

Discussion

Diabetic retinopathy is a major complication of DM, which remains a leading cause of visual loss in working-age populations [14]. Multiple metabolic pathways have been implicated

Table 1: Serum levels of interleukin 1 β and interleukin 18 in patients with diabetic retinopathy.

Test parameters	Groups of subjects		
	Control group (n=30)	Group 1 (n=70)	Group 2 (n=60)
IL1 β pg/mL	1.59 ± 0.05	1.52 ± 0.05 $p > 0.05$	2.28 ± 0.05 $p < 0.05$ $p_1 < 0.05$
IL18 pg/mL	365.0 ± 5.0	368.0 ± 5.0 $p > 0.05$	410.0 ± 5.0 $p < 0.05$ $p_1 < 0.05$
CRP mg/L	2.90 ± 0.3	3.60 ± 0.1 $p < 0.05$	2.95 ± 0.1 $p > 0.05$ $p_1 < 0.05$

Note: p = the probability of differences compared with the control group; p_1 = the probability of differences compared with the patients of Group 1.

in hyperglycemia-induced vascular damage including advanced glycation end products (AGEs) accumulation. AGEs bind to macrophage receptors, which in turn, secrete cytokines that provoke the development of immune responses [4]. Expression levels of pro-inflammatory cytokines such as TNF α , IL6, IL8, and IL1 β were correlated with the severity of diabetic retinopathy [15, 16].

We have found that levels of IL1 β and IL18 were significantly increased in patients with diabetic retinopathy who did not use insulin. As for insulin-dependent patients with diabetic retinopathy, the levels of the investigated pro-inflammatory cytokines were within normal range.

According to Mu Z.P. and co-authors, circulating monocytes in diabetic patients exhibit an excessive inflammatory response to gram-negative bacterial lipopolysaccharides, releasing a large number of pro-inflammatory cytokines, such as IL1 β and TNF α [17]. On the other hand, the metabolic changes that develop in hyperglycemia can directly affect the lymphocyte immunometabolism [18]. Patients with DM are found to have impaired adhesion and cooperation of immunocompetent cells, which results in their increased activation, effect or functions, and migration to the sites of inflammation in the vascular wall. The expression of adhesive molecules in the endothelium and lymphocytes is the initial stage of their recruitment into the site of inflammation, accompanied by the emergence of a wide range of immunological reactions, the latter being essentially protective in nature, but causing necrotic or dystrophic changes secondary to their long duration or intensity. Constant autoactivation of immunocompetent cells leads to an increase in spontaneous production of pro-inflammatory cytokines, which play a key role in diabetes-associated ocular damage with subsequent progression to more severe diabetic retinopathy.

The role of IL1 β in T2D pathogenesis has been a recent topic of interest in the research community. There are data that hyperglycemia induces IL1 β production by β -cells, leading to apoptosis of these cells [9]. Studies report the increase in the levels of IL1 β receptor antagonist to be associated with the risk of developing T2D in the population [10]. These changes can be found more than 10 years prior to the clinical

onset of DM [8] and are associated with insulin resistance. Other studies have shown that hyperproduction of the IL1 β receptor antagonists may predict the risk of cardiovascular complications [19].

The studies in recent years [20, 21] suggest a positive effect of IL1 inhibition on the clinical course of DM. The specific effects include decreasing glucose levels and acute markers of inflammation, and improved functioning of pancreatic β -cells. Notably, these positive effects may persist for prolonged periods of time after completion of treatment with IL1 inhibitors.

Also there are data that point out the importance of inhibiting IL1 in relation to controlling microvascular complications of DM, such as nephropathy, neuropathy, and retinopathy, which may not be directly associated with pronounced hyperglycemia [22].

Either directly or via oxidative stress, IL18 may disrupt endothelial function or stimulate proliferation of vascular smooth muscle cells with the resulting characteristic vascular changes [23]. The researchers have found an increase in plasma levels of IL18 in patients with type 2 diabetes and in patients with metabolic syndrome [8]. Their results suggest a close correlation between IL18 activity and the components to the metabolic syndrome [24]. An increase in circulating IL18 was found in patients with T2D and a prospective study has found elevated IL18 levels to precede the development of T2D [22, 23]. Some other studies demonstrate the association between pro-inflammatory cytokines and obesity, dyslipidemia, insulin resistance and hypertension [8, 9].

The principal biological function of CRP, as well as that of any acute phase protein, is to stimulate immune responses. CRP synthesis is triggered and controlled by a number of relevant mediators, including interleukins, such as IL1 and IL6. By enhancing the synthesis of glucocorticoids, activated interleukins cause leukocytosis, have pyrogenic properties and activate complement cascade and coagulation [8, 25]. Low levels of constantly circulating CRP are not viewed as a specific inflammatory marker because, in addition to induction of acute phase protein

synthesis, interleukins have other inherent functions, which are not associated with acute inflammation [26].

Elevated CRP levels have been implicated in the development of T2DM [27]. Emerging laboratory and epidemiological data now link inflammation and CRP to insulin resistance [28]. Insulin has the capacity to reduce the production of CRP and fibrinogen. Thus, in a setting of insulin resistance, the synthesis of acute-phase proteins is enhanced. As chronic inflammation is part of insulin resistance syndrome, an increase in CRP levels suggests systemic inflammation. There are data which have demonstrated a linear increase in CRP levels with a concomitant increase in metabolic alterations [26].

The researchers have found complement activation and stimulation of expression of adhesion molecules on endothelial surface, as well as binding and modification of lipoproteins (occurring with involvement of CRP) to be suggestive of the initial phase of vascular wall damage and endothelial dysfunction. The destruction of the endothelial glycocalyx layer by CRP was proven by evidences found during in-vivo studies such as elevated levels of hyaluronan in the blood circulation (possibly caused by its erosion from the endothelial cell surface) and decreased number of heparin sulphate staining observed on the aortic endothelium of the rats [28]. Moreover, other factors mediated by CRP which lead to endothelial dysfunction include decrease in the activity of endothelial nitric oxide synthase in the endothelial cells which lead to decreased production of nitric oxide in the concerned cells [29]. Since it is a well-known fact that endothelial dysfunction is the precursor of many diabetic complications such as diabetic retinopathy and diabetic nephropathy, CRP can thus be suspected to be involved as one of the mediators leading to the progression of these diabetic complications [30, 31].

Conclusions

The obtained results suggest that insulin-dependent patients with diabetic retinopathy demonstrate predominance of acute inflammation, which is confirmed by acute increase in

phase marker i.e., C-reactive protein (CRP), and normal levels of pro-inflammatory cytokines (IL1 β and IL18) in blood serum. Patients with diabetic retinopathy who do not use insulin were found to have increased serum levels of IL1 β and IL18 and normal levels of CRP, which suggests the predominance of pro-inflammatory processes (mobilization and activation of cells typically involved in inflammatory process).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Original Research

Determination of Serum Adiponectin Levels in Type 2 Diabetes Patients of the Saudi Population in the Al-Jouf Region

**Badr Alzahrani¹, Muhammad Ikram Ullah¹, Muhammad Atif¹, Ayman Ali Mohammed Alameen^{1,2},
Bayan Mahmoud Alkasabreh¹, Mohammed Al-Ruwili³, Munawir Almohna¹, Alhmidi Alhabbad¹,
Fares Alruwili¹, Abdelhadi Almejabel¹**

¹ Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Al-Jouf Region, Saudi Arabia

² Department of Chemical Pathology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum, Sudan

³ Diabetic Center, King Abdulaziz Specialist Hospital, Ministry of Health, Al-Jouf Region, Saudi Arabia

*Correspondence to: Badr Alzahrani. College of Applied Medical Sciences Jouf University, Al-Jouf province, Saudi Arabia.
E-mail: baalzahrani@ju.edu.sa. Phone: +966553139444.

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Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is currently considered a worldwide and national health problem. Adiponectin is a hormone that is secreted by the adipose tissue and regulates glucose and lipid metabolism. This study aimed to determine the serum adiponectin levels in T2DM patients of the Al-Jouf region of Saudi Arabia. **Material and Methods:** We recruited 65 diabetes patients and 50 healthy control subjects. Serum levels of fasting blood glucose (FBG), total lipid profile levels, adiponectin, and insulin were measured in all subjects. **Results:** The comparison of biochemical parameters, including fasting glucose, insulin, T-cholesterol, and low-density lipoprotein (LDL), were significantly higher in patients than controls. The mean body mass index (BMI) and the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index were significantly lower in controls compared to the T2DM cases. Considerably reduced serum adiponectin ($p < 0.01$) levels were observed in patients rather than controls. Further, a base comparison of the serum adiponectin demonstrated notably reduced adiponectin ($p < 0.01$) levels in females compared to males. **Conclusions:** Serum adiponectin levels were reduced in people with diabetes compared to controls. In comparison to males, females showed reduced adiponectin levels.

Keywords: Diabetes mellitus, insulin resistance, metabolic syndrome, adiponectin, Al-Jouf.

Introduction

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia arising from insulin deficiency, insulin insufficiency, or both. Many factors contribute to the development of diabetes, ranging from autoimmune antibodies against the β cells of the pancreas, causing insulin deficiency to abnormalities related to defects that cause insulin resistance within cells [1]. Worldwide, diabetes mellitus is rapidly becoming more prevalent. Recent studies indicate that approximately 382 million people

have diabetes, with an overall prevalence of 8.3%. Saudi Arabia has one of the highest levels of diabetes [2].

Insulin resistance, or type 2 diabetes, is not a single disease but rather a group of diseases; that is, it is a condition with different characteristics and pathophysiologies but identical symptoms and outcomes. Type 2 diabetes mellitus (T2DM) is a disorder with a combined etiology of insulin resistance and insulin deficiency [3]. Obesity is a common risk factor responsible for developing different metabolic syndromes, including T2DM [4, 5]. Obesity causes different metabolic



abnormalities that result in an increased hepatic glucose output and decreased skeletal muscle, liver, and adipose tissue sensitivity to insulin - the main processes closely related to the pathogenesis of T2DM [6].

Adiponectin is a hormone similar to glycoprotein that is mainly produced by adipose tissues [7, 8]. This molecule is secreted into circulation in three main isoforms: low-molecular-weight (LMW), middle-molecular-weight (MMW), and high-molecular-weight (HMW) [7]. The most biologically active molecule of adiponectin in humans is the high-molecular-weight isoform.

Lower levels of this isoform mostly correlate with the occurrence of T2DM [9–11]. Adiponectin operates through three receptors: AdipoR1, AdipoR2, and T-cadherin. AdipoR1 is expressed at higher levels in muscular tissue, but AdipoR2 is expressed in the hepatocytes. Several studies mentioned the presence of AdipoR1 in different organs, including endothelial cells, cardiac muscle [12, 13], and pancreatic beta cells [14]. Obesity decreases adiponectin levels, resulting in insulin action resistance [15]. Various studies have observed a decrease in adiponectin levels in T2DM [16–18].

The objective of the present study was to determine the serum adiponectin levels in T2DM patients of the Al-Jouf region of Saudi Arabia.

Material and Methods

Ethical declaration

Ethical approval was received from the Local Committee of Bioethics (LCBE), Jouf University (Sakaka, Saudi Arabia). Informed consent to participate in the study was obtained from all patients and control subjects.

Study design and patients

A total of 65 unrelated Saudi diabetic patients in the Al-Jouf region participated in this research. Patients were selected from the King Abdul Aziz Specialist Hospital, Sakaka, Al-Jouf.

The World Health Organization (WHO) criteria were used to diagnose all diabetic patients. Demographic data were collected for all participants using a questionnaire that included questions about weight, height, family history of diabetes, and insulin intake. Body mass index (BMI) was calculated by dividing the body weight by height squared (kg/m^2). BMI values of 25–30 kg/m^2 were considered overweight, and values above 30 kg/m^2 were considered obese [19]. For control subjects, 50 healthy individuals participated in this research. All control subjects had no diabetic family history, no history of taking insulin, and healthy BMIs.

Collection of samples

Venous blood was collected from all subjects after an overnight fast of at least ten hours in an aseptic environment using a sterile yellow cap tube containing gel for serum separation. Serum was separated through centrifugation of clotted blood at 4,000 rpm for 10 minutes within three hours of blood collection and used for the estimation of adiponectin and insulin levels, as well as other research requirements.

Research investigations

1. The GOD-PAP method was used for the estimation of fasting blood glucose (FBG).
2. Fasting insulin was measured by enzyme-linked immunosorbent assay (ELISA) using a Bioassay Technology Laboratory kit (Shanghai, China).
3. Insulin resistance was calculated by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) using the following formula: $\text{HOMA index} = (\text{fasting insulin } (\mu\text{U}/\text{ml}) \times \text{fasting plasma glucose } (\text{mg}/\text{dl}) / 405$ [20].
4. Plasma adiponectin levels were measured by ELISA using a Bioassay Technology Laboratory kit (Shanghai, China).
5. Total lipid profile levels:
 - a. Total cholesterol and triglycerides levels were estimated using the CHOD-PAP and GPO-PAP methods, respectively.

- b. High-density lipoprotein (HDL) was measured using the direct homogeneous enzymatic colorimetric method, whereas low-density lipoprotein (LDL) was determined by the Friedewald formula [21].

$$6. \text{ LDL} = \text{total cholesterol} - (\text{HDL} + \text{TG}/5)$$

Statistical analysis

Results were entered and analyzed statistically using the GraphPad Prism software. An independent t-test was conducted to compare the mean $\pm$ SEM of the control and patient groups; a p-value ≤ 0.05 was considered statistically significant.

Results

A total of 115 subjects, including 65 diabetic patients and 50 healthy controls, was investigated in this study. Of the total participants, 47.8% were female, and 52.2% were male (Table 1). The mean age for T2DM cases was 50.83 ± 1.16 , while the mean age for the controls was 42.73 ± 0.5354 .

Different anthropometric and biochemical parameters were compared between the groups (Table 2). The mean BMI was lower in the controls (23.82 ± 0.6385) than in the T2DM cases (31 ± 0.8469), which was statistically significant

($p < 0.01$). The HOMA-IR was observed to be significantly higher in T2DM patients than in the controls ($p < 0.0001$).

Biochemical parameters, including fasting glucose, insulin, and lipid profiles, were significantly higher in the T2DM cases compared to the controls. The means for fasting glucose in patients and controls were 189.9 ± 7.694 and 89.9 ± 1.871 , respectively ($p < 0.0001$). The mean insulin levels were found to be greater in T2DM patients (19.8 ± 2.343) compared to the control groups (10.23 ± 0.937). However, serum triglyceride and serum HDL-cholesterol were not significantly different between the two groups ($p > 0.05$).

The adiponectin serum levels had a mean of 4.837 ± 0.321 and 7.336 ± 1.081 in T2DM cases and controls, respectively. Significantly reduced serum adiponectin ($p < 0.01$) was observed in T2DM patients as opposed to controls (Figure 1). Further, a base comparison of serum adiponectin demonstrated that adiponectin ($p < 0.01$) was significantly reduced in females rather than males.

Table 1: Distribution of study groups according to gender.

Gender	Groups		Total N (%)
	Controls N (%)	T2DM cases N (%)	
Males	30 (60)	30 (46)	60 (52.2)
Females	20 (40)	35 (54)	55 (47.8)
Total	50 (100)	65 (100)	115 (100)

Table 2: Comparison of descriptive variables between T2DM cases and healthy controls.

Parameters (mg/dl)	Controls	T2DM patients	P-value
Age (years)	42.73 ± 0.5354	50.83 ± 1.16	$< 0.01^*$
BMI (kg/m^2)	23.82 ± 0.6385	31 ± 0.8469	0.0024^{**}
FBG (mg/dl)	89.9 ± 1.871	189.9 ± 7.694	$< 0.0001^{****}$
Insulin ($\mu\text{IU}/\text{ml}$)	10.23 ± 0.937	19.8 ± 2.343	$< 0.0001^{****}$
HOMA-IR index	2.408 ± 0.246	8.534 ± 1.083	$< 0.0001^{****}$
TG (mg/dl)	139 ± 7.603	180.9 ± 13.59	0.0768
T-cholesterol (mg/dl)	145.5 ± 3.904	179.7 ± 9.272	0.0319^*
HDL (mg/dl)	45.13 ± 1.158	46.04 ± 2.141	0.7980
LDL (mg/dl)	72.53 ± 4.287	115.6 ± 7.458	0.0004^{***}

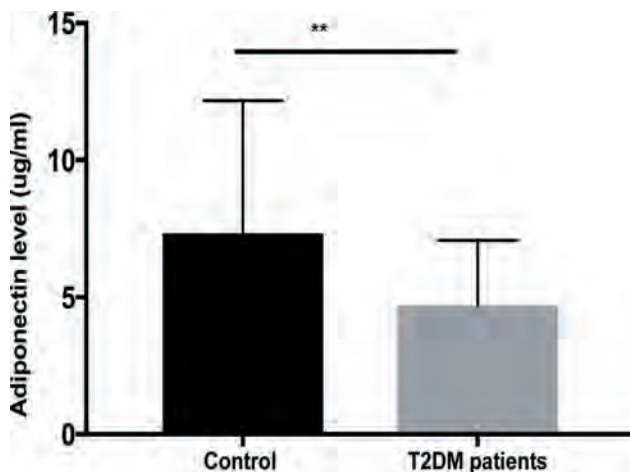


Figure 1: Serum adiponectin levels in T2DM cases and controls.

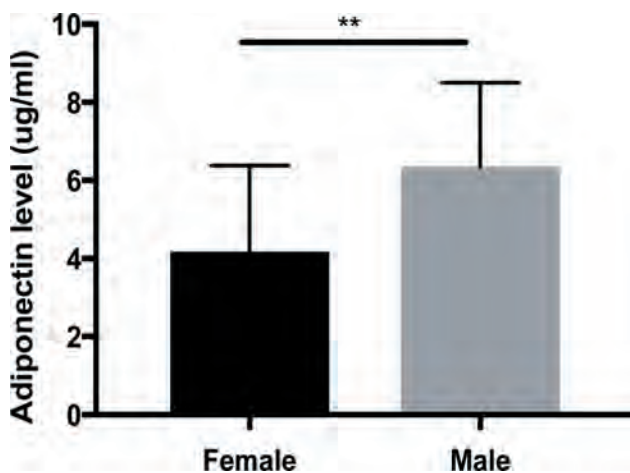


Figure 2: Comparison of serum adiponectin levels in females and males with T2DM.

The mean serum adiponectin was 6.355 ± 0.595 in males and 4.179 ± 0.343 in females (Figure 2).

Discussion

T2DM is the most prevalent form of diabetes mellitus and causes enormous health and economic consequences [22]. Reliable diagnostic tests for T2DM include the FBG test, the oral glucose tolerance test, and the glycated hemoglobin. However, there remain limitations in the use of these tools for the diagnosis of T2DM. Therefore, additional diagnostic biomarkers of T2DM are being explored. Adipocytokines regulate the sensitivity of peripheral tissues to insulin, glucose, and lipid metabolism; they modulate

appetite, angiogenesis, blood pressure, energy expenditure, homeostasis, the axis of endocrine, and the reproductive systems [23]. Some studies documented the role of adiponectin in the pathophysiology of T2DM and metabolic syndrome [24].

In the present study, the levels of serum adiponectin were lower in the T2DM cases than in the healthy controls. The results of our study are comparable to those of previous studies in which reduced adiponectin levels were demonstrated to be independent of age, gender and BMI in T2DM patients [25–28]. Conversely, some studies describe high adiponectin concentrations in T2DM participants [29]. The major factor contributing to this difference is the underlying insulin resistance in type 2 diabetic patients [28, 30]. Insulin is the hormone that mediates the secretion of proteins, including adiponectin, from the adipose tissue and this adiponectin provokes the sensitivity of insulin [31].

In our study, BMI and HOMA-IR were determined and differed significantly between the controls and the T2DM cases. The HOMA-IR was higher in people with diabetes, establishing the development of insulin resistance. Previous studies have reported low adiponectin concentrations to be associated with the risk of obesity [28]. A negative correlation between BMI and adiponectin levels has also been established [25, 32, 33]. The essential role of adiponectin is to act as a hypoglycemic and antidiabetic agent [31]. Therefore, the reduced adiponectin levels in T2DM might enhance the development of insulin resistance and pancreatic dysfunctions [28]. However, the relationship between decreased adiponectin concentration and the effect of insulin resistance remains unclear.

Our results showed that the mean adiponectin levels were significantly lower in diabetic females than males. These results are comparable to those of previous studies reporting reduced adiponectin levels in diabetic females as opposed to males [34]. However, some studies documented no change in adiponectin concentrations in diabetic subjects [28].

In the present study, lipid profile tests, including total cholesterol and LDL cholesterol, were significantly higher in people with diabetes than in non-diabetics, while the presence of

serum triglyceride and serum HDL-cholesterol was found to be non-significant between the groups. Previous research supports the results of the current study [28]. The association between T2DM and dyslipidemia has been well documented [35]. However, the previous research has presented variable correlations between serum adiponectin and lipid profiles. Mostly, there is an inverse relationship between triglyceride and adiponectin [36, 37]. Some studies have demonstrated the correlation of reduced adiponectin with lower HDL and raised LDL cholesterol [37, 38]. Recently, a study from India revealed that a low adiponectin level is correlated with dyslipidemia [25].

Conclusion

Anthropometric and biochemical profiles are significantly associated with the risk of T2DM. Serum adiponectin levels were reduced in diabetics as opposed to controls. In females, rather than males, reduced adiponectin was associated. It is suggested that variable concentrations of adiponectin can lead to an increased risk of obesity, T2DM, insulin resistance, and metabolic syndrome. Further, the development of adipocytokines as biomarkers may be helpful in managing the risks of metabolic syndrome complications.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Inflammatory Atherosclerosis Risk and Lipid Ratios Markers in Type 1 Diabetic Patients: The Role of Ketosis as a Metabolic State

Walid Hassene Hamri^{1,2}, Mustapha Diaf^{1*}, Mohamed Hadj Habib^{3,4}

¹ Department of Biology, Faculty of Natural and Life Sciences, Djillali Liabes University, Sidi-Bel-Abbes, Algeria

² Laboratory of Molecular Microbiology Proteomics and Health, Djillali Liabes University, Sidi-Bel-Abbes, Algeria

³ Department of Medicine, Faculty of Medicine Mourad Taleb, Djillali Liabes University, Sidi-Bel-Abbes, Algeria

⁴ Endocrinology-Diabetology Department, Abdelkader Hassani University Hospital Center of Sidi-Bel-Abbès, Algeria

*Correspondence to: Dr. Mustapha Diaf, PO Box 89, Department of Biology, Faculty of Natural and Life Sciences, Djillali Liabes University of Sidi-Bel-Abbes (220000), Algeria. E-mail: diafmustapha@gmail.com. Phone: 00213 696495465.

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Abstract

Introduction: Epidemiological inquiries on the subclinical atherosclerotic inflammatory disease in ketosis-onset type 1 diabetes (T1D) are scarce. Our objective was to evaluate the risk of developing atherosclerosis via lipid ratios by comparing two groups of patients according to their ketosis status. **Material and Methods:** We performed a comparative retrospective study, including 351 patients (13–74 years) with confirmed T1D divided into the following groups: patients with inaugural diabetic ketosis (IDK) vs. patients with diabetic ketosis (DK). Clinical, biological and pathophysiological patients' data were compared. Main lipid ratios have been assessed according to the patients' ketosis status and gender. The multivariate logistic regression test was applied to evaluate the association of lipid ratios quartiles with the diabetic ketosis status. **Results:** Our study showed a slight predominance of males (50.43%), with an average age of 25.77 years. Comparing to IDK, DK patients had a higher body mass index. No significant differences were observed between fasting plasma glucose and HbA1C and lipid levels. The results showed that the third (OR= 2.14 [0.81–5.61]; p=0.12) and the fourth quartiles (OR= 0.9 [0.35–2.33]; p=0.83) of TC/HDL ratio were higher in both groups. We noticed higher concordant values on the last two quartiles (third and fourth) of the LDL/HDL ratio with p values of 0.07 and 0.39, respectively. In both groups, TC/HDL and TG/HDL were slightly higher in males compared to female T1D patients except for the LDL/HDL ratio, which showed a slight ascendant median in females compared to males with IDK. **Conclusions:** The prevalence and risk of atherosclerosis inflammatory disease were higher in newly and formerly diagnosed patients with ketosis-onset T1D. Thus, medical awareness initiatives and a routine thorough endothelial function are needed to reduce the prevalence of the risk of atherosclerotic inflammatory disease.

Keywords: Atherosclerosis risk, lipid ratios, ketosis, type 1 diabetes.

Introduction

In a global picture, the number of patients diagnosed with diabetes increased up to 463 million among the population aged between 20 and 79 years. If these tendencies remain by 2045, 700.2 million people will be living with diabetes [1].

Multiple epidemiological investigations have shown that the incidence rate of type 1 diabetes (T1D) has been escalating to 2–5% annually [2]. This increase in the number

of cases is particularly felt in low and middle-income countries, and children are not spared by the spread of the disease [3].

In Algeria, the disease's prevalence is continuously increasing, which will lead to a financial burden for the government budget [4].

Unlike other types of diabetes, T1D is characterized by immune-interfered destruction of pancreatic β cells, which negatively affects the secretion of insulin [5]. This leads over time to major microvascular complications like neuropathy, retinopathy, and nephropathy,



among other macrovascular complications like peripheral artery and cardiovascular diseases [6].

Several studies show that up to 40% of patients with new-onset T1D have diabetic ketoacidosis (DKA) [7, 8], which is a serious, potentially deadly T1D complication [9]. The mortality rate associated with DKA depends on the experience of the treating hospital in dealing with this condition [10]. Thus, it is crucial that patients detect DKA and get medical care immediately.

Although the metabolic dysfunction and autoimmune implication in ketosis T1D have been well documented [11, 12], slightly is known regarding the prevalence, clinical and biological features of atherosclerosis in ketosis T1D, which is defined as a persistent, progressive inflammatory process that initiates with lipid deposits and fatty streaks on the arterial intima leading to atherosclerotic plaques [13].

T1D is often associated with the early manifestation of atherosclerosis, representing the morphological basis for macrovascular diabetic complications and shows certain specific features that result from different proportions of risk factors [14].

Recent studies have shown that patients with T1D are at higher risk for endothelial dysfunction and atherosclerosis [15, 16]. However, few studies have inspected the possibilities of influencing and detecting the atherosclerotic process in subjects with ketosis T1D.

Thus, the objective of this scientific study is to assess and to compare the biological, clinical and pathophysiological characteristics of T1D patients with inaugural diabetic ketosis and with diabetic ketosis in order to evaluate the potential atherosclerosis risk, as a preliminary cardiovascular complication, by evaluating blood lipid ratios in a population from northwestern Algeria.

Material and Methods

Population and study design

We underwent a transverse retrospective observational study that involved all patients newly and formerly diagnosed with T1D and

aged from 13 to 74 years. The study was carried out between January 2009 and December 2019 at the Diabetes-Endocrinology Department of the University Hospital Center in Sidi-Bel-Abbes, northwestern Algeria. Patients' medical records were reviewed for medical history, symptoms and signs, biochemical parameters, and complications of the diabetic disease. All T1D patients with Inaugural Diabetic Ketosis (IDK) and Diabetic Ketosis (DK) with complete and adequate medical records were involved in the analysis. Diabetic ketoacidosis (DKA) was defined according to the latest biochemical criteria for the DKA identification published by the American Diabetes Association (ADA) [17]. However, T1D patients without confirmed ketosis were excluded.

For all patients, anthropometric parameters (body weight, body mass index "BMI" and waist circumference) were available on the patient's medical record. Blood pressure was determined by a sphygmomanometer in a supine position followed by a second measurement (after a few minutes) in a standing position. Hypertension was defined by systolic blood pressure (SDP) of 140 mmHg and diastolic blood pressure (DBP) of about 90 mmHg or more. The latest biochemical assessment including fasting blood glucose, glycated hemoglobin (HbA1c), hemoglobin, urea, creatinine and lipid parameters, namely total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglycerides (TG), were taken from patients' medical records. Moreover, lipid ratios as indicators of atherogenic risk were calculated - TC/HDL, LDL/HDL, and TG/HDL.

Ethical considerations

Complete confidentiality of vital patient information was maintained for ethical purposes, and ethical approval was obtained from the department in which the study was carried out.

Statistical analyses

Descriptive analyses were conducted after the data were summarized using percentages and

relative frequencies for qualitative variables and means $\pm$ SD with its respective 95% confidence intervals (95% CI) for continuous variables.

Qualitative categorical variables were compared using the Chi-square test, while quantitative variables were compared using the Student t-test. Significant differences were maintained when the p-value was less than or equal to 0.05 ($p < 0.05$).

Multivariate logistic regression was applied to evaluate differences of categorical variables while adjusting for serum lipid ratios and exploring their clinical usefulness to identify the atherosclerosis risk in T1D patients with IDK or DK. All data were computed and analyzed via the SPSS software (SPSS 22, IBM Corporation; Chicago, IL. August 2013).

Results

The basic characteristics of the studied patients are summarized in Table 1. A total of 351 patients with type 1 diabetes (50.43% males and 49.57% females) were admitted to the University Hospital Center from January 2009 to December 2019. Among all the involved patients, 155 patients (44.2%) presented DKA at first diagnosis of diabetes (inaugural diabetic ketosis "IDK"), while 196 DKA patients (55.8 %) had long-established T1D (diabetic ketosis "DK").

At diagnosis, the age of patients was more than 13 years old in all of them with a mean age of 25.77 years. Meanwhile, the mean age of patients with diabetic ketosis (DK) was

Table 1: Basic characteristics of the participants.

Variables	All Patients n=351	Inaugural Diabetic Ketosis n=155	Diabetic Ketosis n=196	p value*
Gender, n (%)				
Male	177 (50.43)	100 (64.52)	77 (39.29)	<10 ⁻³
Female	174 (49.57)	55 (35.48)	119 (60.71)	<10 ⁻³
Age groups, years				
[13–19]	102 (29.10)	53 (34.20)	49 (25.00)	<10 ⁻³
[20–29]	148 (42.20)	75 (48.40)	73 (37.20)	
[30–39]	81 (23.10)	27 (17.40)	54 (27.60)	
[40–49]	15 (4.30)	0 (0.00)	15 (7.70)	
[50–59]	3 (0.90)	0 (0.00)	3 (1.50)	
≥ 60	2 (0.60)	0 (0.00)	2 (1.00)	
Smoking history, n (%)				
Male	53 (15.10)	34 (21.93)	19 (9.69)	<10 ⁻³
Female	–	–	–	–
Prevalence of weight categories, n (%)				
Underweight, BMI <18.5 Kg/m ²	119 (33.90)	67 (43.22)	52 (26.53)	0.009
Normal weight, BMI=18.5–25.0Kg/m ²	163 (46.40)	70 (45.16)	93 (47.45)	
Overweight, BMI=25.0–29.9Kg/m ²	20 (5.70)	5 (3.22)	15 (7.65)	
Obesity, BMI ≥30 Kg/m ²	9 (2.60)	5 (3.22)	4 (2.04)	
Precipitating factors, n (%)				
Angina	17 (4.80)	9 (5.80)	8 (4.08)	0.45
Influenza syndrome	33 (9.40)	7 (4.50)	26 (13.27)	0.005
Pregnancy	13 (3.70)	0 (0.00)	13 (6.63)	0.001
Urinary infection	73 (20.80)	25 (16.14)	48 (24.49)	0.05
Dental abscess	8 (2.28)	1 (0.66)	7 (3.57)	0.06
Insulin omission	16 (4.57)	0 (0.00)	16 (8.16)	<10 ⁻³
Diabetic foot	3 (0.90)	0 (0.00)	3 (1.53)	0.12
Alcohol abuse (in males)	10 (2.84)	7 (4.51)	3 (1.53)	0.09
Unknown	178 (50.71)	106 (68.39)	72 (36.74)	–

(continued)

Table 1: Continued

Variables	All Patients n=351	Inaugural Diabetic Ketosis n=155	Diabetic Ketosis n=196	p value*
Other associated diseases, n (%)				
Low visual acuity	44 (12.50)	8 (5.16)	36 (18.37)	$<10^{-3}$
Diabetic retinopathy	13 (3.70)	0 (0.00)	13 (6.63)	$<10^{-3}$
Hypertension	9 (2.60)	0 (0.00)	9 (4.59)	$<10^{-3}$
Hypothyroidism	13 (3.70)	2 (1.29)	11 (5.16)	0.03
Hyperthyroidism	6 (1.70)	4 (2.58)	2 (1.02)	0.26
Anemia	76 (21.70)	24 (15.48)	52 (26.53)	0.01
Gastroenteritis	8 (2.30)	1 (0.65)	7 (3.57)	0.06
Symptoms and signs, n (%)				
Signs of dehydration	87 (24.80)	44 (28.38)	43 (21.94)	0.16
Abdominal pain	67 (19.10)	18 (11.61)	49 (25.00)	0.001
Vomiting and nausea	98 (27.90)	14 (11.61)	84 (42.86)	10^{-3}
Dyspnea	18 (5.10)	7 (9.03)	11 (5.61)	0.64
Transit disorder (diarrhea)	15 (4.30)	2 (1.29)	13 (6.63)	0.01
Epigastric	21 (6.00)	1 (0.65)	20 (10.20)	0.001
Headaches	25 (7.10)	13 (8.39)	12 (6.12)	0.41
Dizziness	27 (7.70)	17 (10.97)	10 (5.10)	0.04
Edema	5 (1.40)	1 (0.65)	4 (2.04)	0.27
Micturition burn	22 (6.30)	6 (3.87)	16 (8.16)	0.1
Cardinal syndrome, n (%)	245 (69.80)	129 (83.23)	116 (5.61)	$<10^{-3}$
Weight loss	349 (99.40)	155 (100.00)	194 (98.97)	0.20
Polyuria-Polydipsia	186 (53.00)	84 (54.19)	102 (52.04)	0.98
Asthenia	258 (73.50)	140 (90.32)	118 (60.20)	0.01
Overeating				
Family history, n (%)				
Hypertension	82 (23.36)	37 (23.87)	45 (22.96)	0.84
Type 1 diabetes	56 (15.95)	22 (14.19)	34 (17.35)	0.42
Type 2 diabetes	195 (55.55)	89 (57.42)	106 (54.08)	0.53
Goiter	20 (5.69)	9 (5.80)	11 (5.61)	0.93

(*) percentages were compared with the Chi-square test, $p \leq 0.05$ was considered as significant; BMI: body mass index.

significantly higher than that of patients with inaugural diabetic ketosis (IDK) (27.83 ± 9.93 years vs. 23.16 ± 5.74 years, $p < 0.001$) (Table 2). The most affected age group by both IDK and DK was the 20–29 age group, with a rate of 42.2%, followed by the 13–19 age group with 29.1%. However, the least affected age group was the >60 years group (Table 1). The overall prevalence of underweight, normal weight, overweight, and obesity was 33.90%, 57.80%, 5.70%, and 2.60%, respectively.

In the current study, multiple factors contributed to DKA, including infection, poor drug compliance, pregnancy, diabetic foot, and alcohol abuse. The most commonly known precipitating factor for DKA was urinary infection 20.8%, followed by the influenza syndrome – 9.4%, angina – 4.8% and insulin omission – 4.57%. Anemia was the most common complication (21.7%), its

frequency being higher in the DK group (26.53%, $p = 0.01$), followed by low visual acuity, diabetic retinopathy, hypothyroidism, and hypertension.

As mentioned in the patients' medical files, the typical symptoms were mostly vomiting and nausea – 27.9%, followed by signs of dehydration – 24.8% and abdominal pain – 19.1% as the presenting symptoms. Other symptoms defined by cardinal syndrome were also present and patients had one or more of them, including polyuria polydipsia, overeating, weight loss, and asthenia.

Family history (grandparents, parents, and siblings) of T1D was present in 15.95% of cases, while the majority (84.6%) reported having a family history of other complications (T2D, hypertension, and goiter).

Laboratory data for the two groups are recapitulated in Table 2. Concerning the

Table 2: Comparison of biochemical characteristics between ketosis patients' subgroups.

Variables	All Patients n=351		Inaugural Diabetic Ketosis n=155		Diabetic Ketosis n=196		pvalue*
	Mean±SD	95% CI	Mean±SD	95% CI	Mean±SD	95% CI	
Mean age (years)	25.77±8.65	24.86–26.67	23.16±5.74	22.25–24.07	27.83±9.93	26.43–29.23	<10 ⁻³
Diabetes duration (years)	5.92±7.57	5.13–6.72	0.11±0.07	0.10–0.12	10.52±7.4	9.48–11.56	<10 ⁻³
Age at 1 st diagnosis (years)	19.95±7.82	19.13–20.78	23.15±5.75	22.24–24.06	17.43±8.31	16.26–18.6	<10 ⁻³
Body weight (Kg)	56.86±12.12	55.51–58.21	57.53±11.66	55.63–59.43	56.26±12.53	54.33–58.19	0.35
BMI (Kg/m ²)	19.96±3.60	19.56–20.36	19.36±3.47	18.80–19.93	20.50±3.63	19.93–21.06	0.005
Waist circumference (cm)	78.37±10.19	76.50–80.23	78.18±10.77	75.29–81.06	78.54±9.72	76.05–81.03	0.84
SBP (mmHg)	110.60±11.66	109.40–111.90	110.80±10.84	109.10–112.50	110.50±12.30	108.80–112.20	0.83
DBP (mmHg)	66.70±8.31	65.80–67.60	67.40±8.21	66.10–68.70	66.20±8.38	65.00–67.30	0.15
Fasting plasma glucose (g/l)	3.76±0.91	3.66–3.85	3.69±0.93	3.54–3.84	3.81±0.89	3.68–3.94	0.22
HbA1c (%)	11.42±2.08	11.16–11.69	11.67±2.12	11.28–12.07	11.22±2.03	10.87–11.56	0.08
Haemoglobin(g/l)	13.04±2.05	12.76–13.31	13.66±2.05	13.26–14.06	12.48±1.89	12.13–12.83	<10 ⁻³
Total cholesterol (g/l)	1.58±0.41	1.52–1.65	1.60±0.44	1.50–1.71	1.56±0.38	1.48–1.65	0.54
HDL-c (g/l)	0.40±0.11	0.38–0.42	0.39±0.08	0.37–0.41	0.40±0.13	0.37–0.44	0.48
LDL-c (g/l)	0.93±0.34	0.87–0.98	0.95±0.35	0.87–1.04	0.90±0.32	0.83–0.98	0.36
Triglycerides (g/l)	1.05±0.65	0.95–1.15	1.08±0.67	0.92–1.23	1.02±0.63	0.88–1.16	0.58
TC/HDL-c	4.01±1.21	3.80–4.21	4.10±1.15	3.82–4.38	3.92±1.26	3.63–4.22	0.39
LDL/HDL-c	2.39±0.98	2.23–2.55	2.51±0.98	2.27–2.75	2.29±0.97	2.06–2.51	0.18
TG/HDL-c	2.82±2.04	2.47–3.16	2.93±1.88	2.47–3.39	2.71±2.18	2.20–3.22	0.52
Creatinine(mg/l)	12.58±9.95	9.75–15.41	10.78±10.24	5.68–15.87	13.60±9.80	10.07–17.13	0.34
Urea (g/l)	0.49±0.38	0.38–0.59	0.37±0.26	0.23–0.50	0.55±0.42	0.40–0.71	0.05

(*) means were compared with independent sample Student's t-test, $p < 0.05$ was considered as significant; SD: standard deviation; CI: confidence interval; HbA1c: glycosylated hemoglobin; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; TG: triglycerides.

anthropometric measurement on admission, there were no significant differences in body weight and waist circumference between the two groups. However, significantly higher BMI ($p=0.005$) was found in DK comparing to IDK T1D patients. No differences are observed in orthostatic blood pressure (Table 2).

Although the glycemic control was poor, as shown by a mean glycated hemoglobin (HbA1c) level above 10%, no significant differences regarding fasting plasma glucose and HbA1c levels (IDK: $11.67 \pm 2.12\%$, DK: $11.22 \pm 2.03\%$, $p=0.08$) between the two groups of diabetes was noted on admission. On the other hand, hemoglobin was lower in DK patients than in IDK patients (12.48 ± 1.89 g/l vs. 13.66 ± 2.05 g/l, respectively, $p<0.001$). Concerning lipid levels, no significant differences were found between the two groups. In contrast, mean levels of lipid ratios were slightly higher in IDK than in DK without significant differences (Table 2).

With respect to renal function, urea mean $\pm$ SD was higher in the longstanding T1D group (0.55 ± 0.42 g/l), with a significant difference among the two groups ($p=0.05$). Meanwhile, no evidence of a difference in creatinine mean $\pm$ SD was found between the two groups ($p=0.34$).

Regarding fluid management in the emergency room, the initial insulin requirement was higher in patients with DK than that of IDK (50.51 ± 23.72 IU vs. 45.57 ± 18.66 IU, respectively, $p=0.03$).

For the whole study population, the multivariate regression between lipid ratios quartiles, as powerful predictors of atherosclerosis, plotted that the third (OR= 2.14 [0.81–5.61]; $p=0.12$) and the fourth quartiles (OR= 0.9 [0.35–2.33]; $p=0.83$) of TC/HDL ratio were higher in both groups as described in Table 3. Furthermore, we noticed higher concordant values in the last two quartiles (third and fourth) of the LDL/HDL ratio with p values of 0.07 and 0.39, respectively.

Table 3: Crude “Odds Ratio” of blood lipid ratios quartiles associated with the type of diabetic ketosis.

Variables	Inaugural Diabetic Ketosis, n=155 Number (%)	Diabetic Ketosis, n=196 Number (%)	Odds ratio (95% CI OR)	*p-value
CT/HDL ratio				
1 st quartile (1.56–3.12)	14 (20.89)	20 (27.78)	Reference	–
2 nd quartile (3.13–3.92)	13 (19.41)	22 (30.56)	1.81 [0.69–4.73]	0.22
3 rd quartile (3.93–4.61)	21 (31.34)	15 (20.83)	2.14 [0.81–5.61]	0.12
4 th quartile (4.62–7.96)	19 (28.36)	15 (20.83)	0.90 [0.35–2.33]	0.83
LDL/HDL ratio				
1 st quartile (0.35–1.81)	17 (25.37)	18 (25.00)	Reference	–
2 nd quartile (1.82–2.25)	13 (19.41)	22 (30.56)	1.51 [0.58–3.91]	0.39
3 rd quartile (2.26–2.84)	17 (25.37)	18 (25.00)	2.41 [0.91–6.36]	0.07
4 th quartile (2.85–5.93)	20 (29.85)	14 (19.44)	1.51 [0.58–3.19]	0.39
TG/HDL ratio				
1 st quartile (1.00–1.40)	14 (20.89)	20 (27.78)	Reference	–
2 nd quartile (1.41–2.16)	18 (26.87)	18 (25.00)	1.51 [0.58–3.91]	0.39
3 rd quartile (2.17–3.68)	17 (25.37)	17 (23.61)	1.05 [0.41–2.68]	0.90
4 th quartile (3.69–13.58)	18 (26.87)	17 (23.61)	1.05 [0.41–2.72]	0.90

(*) multivariate logistic regression significant at $p = 0.05$; CI, confidence interval; OR, Odd ratio; Q, quartiles; TC: total cholesterol; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; TG: triglycerides.

Likewise, the fourth quartile of the TG/HDL ratio disclosed an Odd ratio of 1.05 [0.41–2.72] ($p=0.90$).

As shown in Figure 1, in males and females, when comparing lipid ratios between the two groups of diabetic ketosis, TC/HDL and TG/HDL were slightly higher in males compared to females T1D patients. However, this was not the case for the LDL/HDL ratio, which showed a slight ascendant median in females compared to males with inaugural diabetic ketosis.

Besides, higher TC and LDL levels were observed in females compared to males with IDK. Meanwhile, males with DK had high levels of TC and LDL compared to female patients. Similarly,

TG levels were higher in males compared to females with IDK.

Discussion

Ketosis is considered a trait of T1D and one of its most serious acute complications. Its annual incidence is around 4.6 to 8 episodes/1000 diabetic patients [18]. This complication is characterized by increased production of ketones and glucose in the liver precipitated by a total or relative lack of insulin and an increase in catabolic hormone rates [19–21]. In parallel, T1D patients

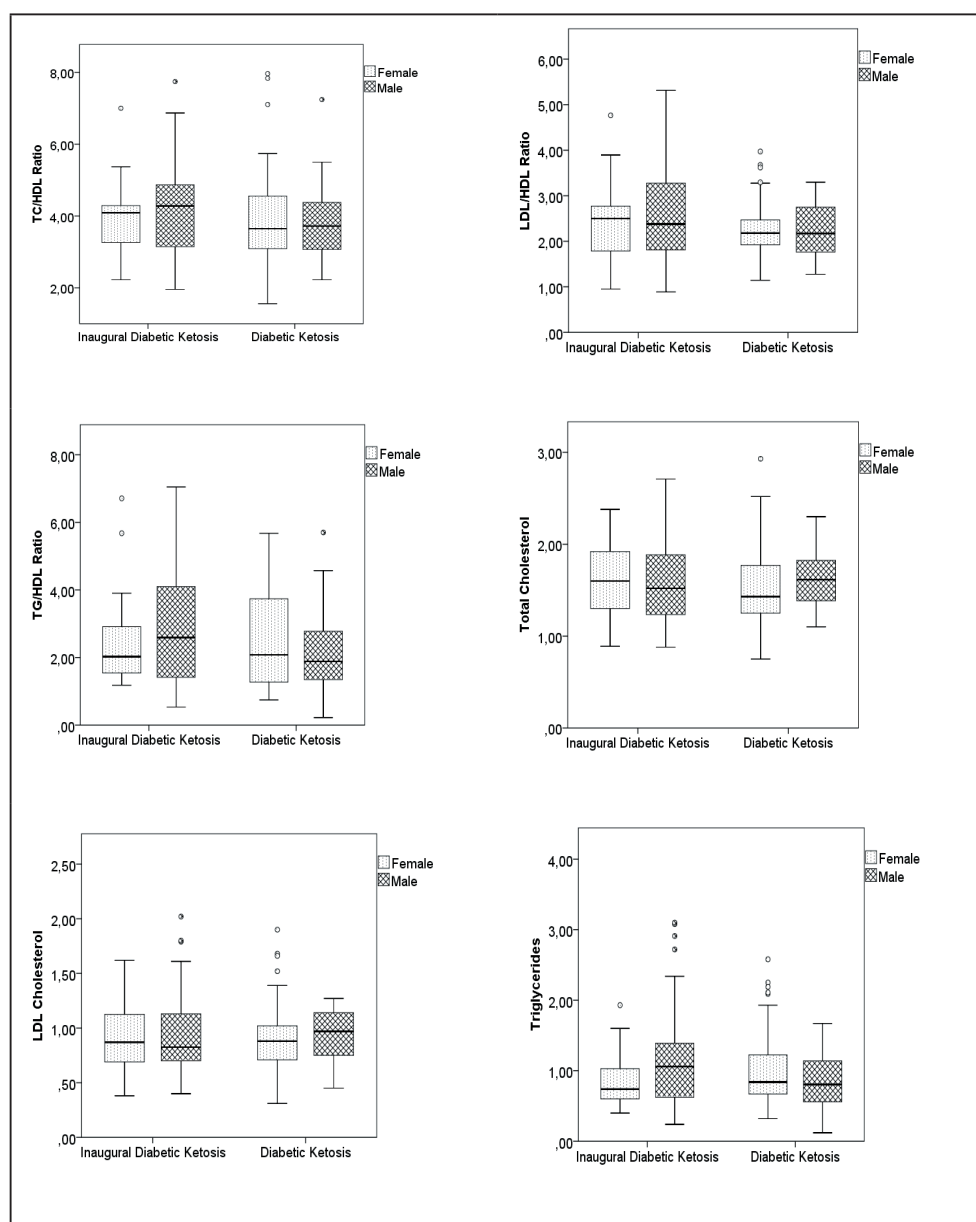


Figure 1: Comparison of lipid ratios levels between patients with Inaugural Diabetic Ketosis and Diabetic Ketosis according to the patients' gender.

are prone to lipid disorders (mainly qualitative abnormalities of the lipoproteins) that can subsequently lead to the onset of atherosclerosis. Hyperglycemia and peripheral hyperinsulinemia, which are likely to play a role in these complications, result from the administration of subcutaneous insulin [22].

The objective of the current study was to evaluate, in T1D patients, the risk of developing atherosclerosis (via lipid ratios) by comparing two groups of patients according to their ketosis status (patients with inaugural diabetic ketosis vs. patients with diabetic ketosis).

Our study's preliminary consequences displayed significant effects of gender, age group, active smoking status, and patients' corpulence on variances between inaugural diabetic ketosis patients and diabetic ketosis ones. The distribution of patients by gender showed a slight predominance of males over females (50.43% – 49.57%), a sex ratio of 1.01. Taieb *et al.* found that 68% of the population diagnosed with type 1 diabetic ketosis were males versus 32% females with a sex ratio = 2.1 [23]. Our results are in accordance with the same Tunisian study conclusion, where a relatively high proportion of inaugural diabetic ketosis has been described in adolescents and young subjects compared to the older patients [23]. The same observations have been made by Lin *et al.* in Taiwan [24]. Studies suggesting the relationship between type 1 diabetes, diabetic ketosis and body corpulence are still rare and inconclusive. Low *et al.* have reported that lean children and adolescents with DK have often type 1 autoimmune diabetes. In contrast, obese children and adolescents with DK have clinical, metabolic, and immunological features of type 2 diabetes [25]. Our findings disclose that most T1D patients with both inaugural diabetic ketosis and diabetic ketosis are either underweight or normal weight with a higher significant effect of body corpulence ($p=0.009$).

The most common precipitating factors reported in the present study were urinary infection, influenza syndrome, angina, and insulin omission that are significantly more frequent in patients with diabetic ketosis compared to those with inaugural diabetic ketosis. Ahuja *et al.* reported that infections and insulin omission

were the most frequent precipitants factors of DKA [26]. In our series, 4.57% of young patients who had omitted their medications had a family history of omission and/or medication neglecting. One explanation for this behavior may be a patients' misunderstanding of their diabetes management [27].

In our study, vomiting and abdominal pain were the most common presenting symptoms; these results concord with other studies [28, 29]. Moreover, the clinical picture of DK typically involves cardinal syndrome (polyuria, polydipsia, weight loss, asthenia, and weakness).

The diabetic ketosis group required more additional fluid and treatment with the insulin infusion to achieve a ketone-negative status compared with the newly diagnosed group [30].

The comparison between the two ketosis patients' subgroups disclosed some significant differences, especially for mean age, the first age of diabetes diagnosis, and biochemical parameters. The glycated hemoglobin ($p=0.08$) and hemoglobin ($p<0.001$) were higher in patients with inaugural diabetic ketosis compared to those with diabetic ketosis. Similarly, the conventional lipid parameters (TC, HDL, LDL, and TG) and their associated ratios (TC/HDL, LDL/HDL, and TG/HDL) were higher in patients with inaugural diabetic ketosis compared to patients with diabetic ketosis indicating a higher cardiovascular risk in new-onset type 1 diabetic patients. There is lacking direct research evaluating the cardiovascular risk (namely of atherosclerosis) in ketosis onset diabetes (inaugural diabetic ketosis) or ketosis-prone diabetes (diabetic ketosis). In their study, Liu *et al.* showed that patients with inaugural diabetic ketosis do not have absolutely the same clinical characters as patients with diabetic ketosis, and each subgroup showed different rates [31].

Many authors agree that atherogenic or Castelli indexes (LDL/HDL and TC/HDL ratios) are the best indicators of vascular risk compared to the traditional lipid parameters [32]. According to the Helsinki study (1992), the LDL/HDL ratio is the most relevant index for cardiovascular health [33]. Our findings disclosed a higher non-significant atherosclerosis risk (evaluated through lipid ratios) in type 1 ketosis-onset diabetics than in

type 1 diabetic ketosis patients. The same results were reported by Wang et al. [34].

In the present study, all participants were categorized into lipid ratios quartiles, from the highest quartile (fourth) to the lowest one (first) as reference. The comparison of lipid ratios quartiles between patients with inaugural diabetic ketosis and those with diabetic ketosis exposed non-significant differences for all the compared quartiles. Whereas, the Odds ratios increased significantly from the first to the third quartiles of the three studied lipid ratios, regardless of the ketosis subgroup. Our results are in accordance with the conclusions of the Framingham study (2006), where increasing coronary heart disease proportions were observed when the TC/HDL ratio fluctuated from the lower to the higher tertiles [35].

The explanation of DK's role in the inflammatory cascade in diabetic patients has been the subject of several works [36]. It is currently clearer that the acceleration of LDL oxidation, hyperketonemia, and the increased risk of atherosclerosis and CVD in diabetic patients result from the high levels of oxidative stress experienced by these patients. It has also been reported that DK can be independently associated with cardiovascular risk despite the absence of a metabolic history or known sensitivities to cardiovascular pathology [37]. In addition, it has been stated that the elevation level of ketones is proportional to the severity of congestive heart failure [38]. In our study, both patients with inaugural diabetic ketosis and diabetic ketosis have a higher to moderate risk of developing atherosclerosis according to their lipid ratios expressed by quartiles.

Regarding the effect of gender difference on the clinical characteristics and outcomes of diabetic ketosis patients, the availability of data is still very limited. These gender differences must be recognized and taken into account for optimal treatment and diabetes prevention [39]. Our results point out that the TC/HDL ratio was beyond the therapeutic target of 3.5 recognized by several authors for patients with inaugural diabetic ketosis and diabetic ketosis [40]. Likewise, the values of this ratio for men were often high compared to women. The LDL/HDL ratio values were higher in women patients with inaugural

diabetic ketosis and almost identical between the two sexes in patients with diabetic ketosis. However, in all studied patients, LDL/HDL and TG/HDL levels were within the normal ranges of 2.5 and 3, respectively [40, 41].

In this study, we had certain limitations that need to be indicated. First, the observational context of the present investigation does not allow to make a clear, conclusive decision about the atherosclerosis risk in T1D with regard to ketosis. Therefore, further intervention study is preferable to confirm our results. Second, most of the study participants were from the urban area, so this population could not be geographically diverse. Nevertheless, regardless of these limitations, we believe that our conclusions would remain unchanged in the general population despite the study design. The employment of lipid ratios in the evaluation of atherosclerosis risk in T1D patients is a robust method regardless of gender and ketosis status; however, lifestyle factors may undoubtedly change over time. Therefore, data from the present work can be a good support for further research.

Conclusion

Diabetic ketosis or ketoacidosis is the most crucial complication of T1D and can present high morbidity if it is not diagnosed early and managed properly. In patients with type 1 diabetes, the employment of blood lipid ratios for evaluating atherosclerosis threats disclosed a higher risk in both groups of patients with inaugural diabetic ketosis and diabetic ketosis. However, higher frequency and dominance of atherosclerosis risk is observed in the male gender with inaugural diabetic ketosis. Consequently, considering the large prevalence of the associated subclinical conditions, and their consequences for the long-term management, a routinely thorough vascular assessment might be warranted for patients with T1D.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

Anti-inflammatory and antioxidant effects of using alpha-tocopherol in cell culture of the parotid gland under conditions similar to diabetes mellitus

Victor Augusto Ramos Fernandes^{1,4}, Raphael Oliveira Ramos Franco Netto¹, Felipe Lovaglio Belozo³, Eduardo José Caldeira²

¹ Researcher at the Laboratory of Tissue Morphology, Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, São Paulo/Brazil

² Professor of the undergraduate course in Medicine and supervisor of the Doctoral program in Health Sciences, Faculty of Medicine of Jundiaí, São Paulo/Brazil

³ Professor at the Faculty of Physical Education of Faculdades Network, Nova Odessa/SP campus, São Paulo/Brazil

⁴ Centro Universitário Nossa Senhora do Patrocínio, Itu, São Paulo / Brazil

*Correspondence to: Dr. Victor Augusto Ramos Fernandes, PhD, Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, São Paulo/Brazil. E-mail: victorramosfernandes@gmail.com

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Abstract

Background and Aims: Hyperglycemia, one of the most common causes that leads to oxidative damage, is frequently observed in association with inflammatory infiltration mediated by mononuclear phagocytes and epithelial cells present in glandular tissues of diabetic patients. Actions that make it possible to reduce tissue damage caused by the oxidative process inherent in this metabolic disease can become an efficient alternative in helping to maintain the Quality of Life of millions of people. The present study aimed to analyze the effects of the use of alpha-tocopherol in cultures of primary cells of the parotid gland submitted to conditions similar to those that occur in diabetes mellitus (DM). **Methods:** For this, cells from the parotid glands of Balb/C/Unib mice were extracted using the Percoll® protocol. The cells were organized into five groups with different amounts of glucose diluted in the cell culture medium and with the presence or absence of lipopolysaccharide to induce the inflammatory process, as well as the presence or absence of alpha-tocopherol. **Results:** The observed results indicated an increase in cell viability with the use of alpha-tocopherol and reductions of this same variable when in the presence of oxidizing agents. The proteins involved with the mediation of antioxidant protection and inflammation, Nuclear factor erythroid 2-related factor 2 (NRF2), nuclear factor kappa B (NfκB), and glutathione peroxidase were increased in the groups submitted to a hyperglycemic and inflammatory condition, but the same behavior was not observed in the other groups of the experiment. **Conclusion:** It is concluded, therefore, that alpha-tocopherol showed anti-inflammatory and antioxidant effects in this type of cell culture, attenuating the harmful effects of the conditions in which the cells were subjected.

Keywords: Antioxidants, Diabetes Mellitus Inflammation, Oxidants, Primary Cell Culture, Vitamin E.

Background and Aims

Diabetes Mellitus (DM) is classified as a complex metabolic disease that affects the metabolism of carbohydrates, lipids, and proteins [1]. In this sense, there is an increase in accumulated lipoproteins and lipids in diabetic

people, resulting in significant changes in cellular health [2]. In this way, many researchers seek to understand the factors that stimulate the development of diabetes. To do this, they are classified into different types, the most common of which currently are diabetes mellitus type 1 (DMT1) and diabetes mellitus type 2 (DM TII) [3].



Studies indicate that for the development of type 2 diabetes, lifestyle, diet, and physical inactivity are the main stimulating factors [4]. Type 1 diabetes, classified as an autoimmune disease, has no apparent cause for its development, however, genetic, epigenetic, and environmental aspects have strong scientific indications [5]. However, both conditions of diabetes present hyperglycemia as a predominant symptom [1-5]. This symptom stems from an abnormal condition of expression or recognition at the level of insulin receptors [6].

Insulin is an anabolic hormone with the ability to stimulate glucose uptake, mainly by muscle and liver cells. Also, insulin increases protein synthesis and signals the cell's pathways for survival. However, dysfunction in its secretion or its recognition results in a greater presence of glucose in the plasma or the extracellular environment [7, 8]. This presence of glucose, above normal values, leads to an increase in reactive oxygen species (ROS) and, therefore, oxidative stress and, later, inflammation [9, 10].

Thus, the inflammatory condition, established in diabetes, promotes several dysfunctions, such as cell death and parenchymal degeneration and increased gland stroma [11]. The pancreas is the organ frequently injured in this condition, however other glands also suffer from hyperglycemia and the inflammatory condition established by it [12-14]. The parotid gland, for example, produces less saliva, leading to a symptom known as xerostomia (presence of dry mouth), in addition to the tissue impairments already described [15]. In this sense, understanding the hyperglycemic and inflammatory effects on other glands can lead to a deeper understanding of these conditions and their tissue and cellular consequences.

On the other hand, several studies seek to develop efficient treatments in the process of interrupting or reducing the damage caused by oxidative stress or even by the inflammation that occurs through this mechanism induced by hyperglycemia [16-20]. Alpha-tocopherol, a molecule belonging to the family of fat-soluble vitamins (isoprenoids), appears as one of the main substances most studied in this perspective [21, 22]. Recognized as a potent antioxidant agent, it

has been observed in other studies carried out in humans and animals [23-26].

The mechanisms of action of alpha-tocopherol are non-enzymatic, i.e., this vitamin works in a way to prevent reactive oxygen species from capturing electrons from other molecules, especially the phospholipids present in cell biomembranes [25].

However, few studies have observed the effects of alpha-tocopherol *in vitro* conditions [26], and even fewer studies have linked this vitamin to the harmful conditions observed in type 1 diabetes and other autoimmune diseases or that modify the metabolism glucose. The aim of this study was to observe the antioxidant, anti-inflammatory, and cellular cytotoxic effects of alpha-tocopherol in a culture of parotid gland cells submitted to a condition similar to DM.

Material and Method

Experimental design and cell isolation

This study was approved by the ethics committee for research in animal models of the Faculty of Medicine of Jundiaí (opinion No. 154/2016); after approval, 12 mice of the Balb/C/Unib strain were selected for this study. The animals were kept *ad libitum* until the fifth week of life, with a 12/12 light/dark cycle. Upon reaching the predicted week, they were euthanized using injectable ketamine (0.10 ml/body weight), intraperitoneally. The parotid glands of these animals were extracted and the process of cell isolation and cell culture of these glands was sequentially followed.

The cell isolation protocol was performed after the mechanical fragmentation of the tissue, a process performed by the researchers in a sterile environment and equipment with laminar flow appropriate for cell culture. Sequentially, the tissue was immersed in collagenase type 1 diluted in RPMI-1640 culture medium (1 mg/ml, Sigma Aldrich). During the tissue dilution time, the sample was stored in an incubator with a temperature of 37°C and 5% CO₂.

After a period of three hours, the sample was removed from the incubator and subjected to the centrifugation procedure (Eppendorf

centrifuge - model 5804/R) for 10 minutes at a speed of 2000 revolutions per minute, at room temperature. After that, the supernatant was aspirated and the pellet was re-suspended in 5 ml of RPMI-1640 cell culture medium (Sigma Aldrich) plus 10% fetal bovine serum (Sigma Aldrich) and 1% antibiotics (penicillin and streptomycin) so that contamination of the material could be avoided. Once the re-suspension was completed, the material was added slowly to a falcon tube containing Percoll (Sigma Aldrich) in different densities to isolate the different types of cells present in the parotid gland. After the parenchymal cells were separated from the stromal cells, a process performed by the density of Percoll, the epithelial cells were added in cell culture plates to observe their development and proliferation.

Treatment of experimental groups

To observe the action of alpha-tocopherol on the cells of the parotid gland, it was divided into five groups, namely:

- Group I: parotid gland cells without treatment, simulating normal conditions (with low glucose and without supplementary treatments in the culture medium);
- Group II: cells of the parotid gland treated with lipopolysaccharides (100 ng/ml) to stimulate the inflammatory process and the formation of oxidizing agents, to observe the effects of the condition similar to diabetes;
- Group III: cells of the parotid gland treated with lipopolysaccharides (100 ng/ml) and alpha-tocopherol (20 mMol) to observe the effects of the condition similar to diabetes and the action of the anti-inflammatory and antioxidant agent;
- Group IV: cells of the parotid gland treated with anhydrous glucose (4.5 g/L) and lipopolysaccharides (100 ng/ml) to stimulate the hyperglycemic and pro-inflammatory condition, present in type 1 diabetes.

Group V: cells of the parotid gland treated with anhydrous glucose (4.5 g/L) and lipopolysaccharides (100 ng/ml) and alpha-tocopherol (20 mMol) to observe the effects of the condition similar to diabetes and the action of the anti-inflammatory and antioxidant agent.

Tests used to observe cellular effect

To observe the cellular effects caused by the use of the substances described in the above section "treatment of the experimental groups" of the present research, the immunoblotting tests for proteins involved in antioxidant and inflammatory processes were used, as well as the tests to verify the toxicity of alpha-tocopherol in cell culture, primaries of this type.

All tests followed specific protocols based on the recommendations of the manufacturers of the products used in the present study.

Statistical treatment

The statistical treatment used in the present study was Anova one way, taking $p < 0.05$ as statistically significant events. All analyses passed the Bonferroni verification test, assuming the same value for p .

Results

The results obtained in this study were classified in chronological order, presented as they were collected, analyzed and verified, always in triplicates. Initially, the evolution and development of cell culture will be presented, through the alpha-tocopherol cell toxicity test (IC-50). Subsequently, the results of the quantification of proteins involved in the processes of induction of inflammatory state, response and cellular antioxidant defense will be presented.

After obtaining the cells, extracted from the animals' parotid glands, it was possible to observe an adherence of the cells, in culture, from the zero hour until five days after the extraction.

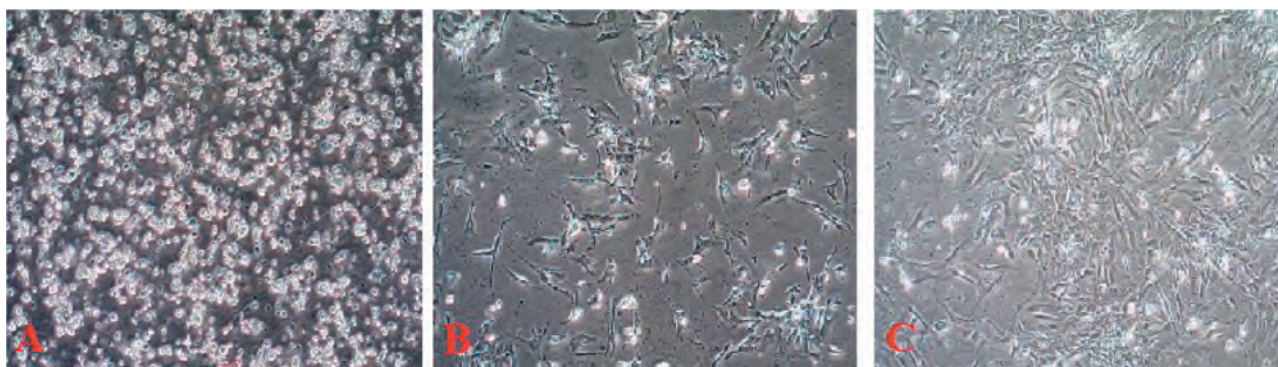
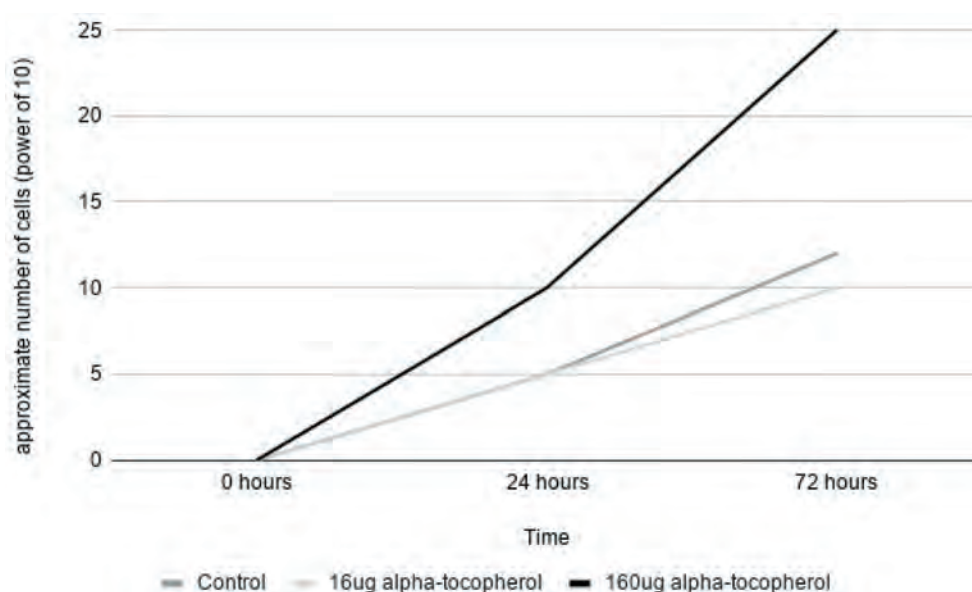


Figure 1: Progression of adherence and cellular development of the cells of the parotid gland. Image A presents immediate conditions after extraction and the Percoll protocol procedure. Image B indicates cell condition after 72 hours in an RPMI-1640 culture medium. Image C shows the condition of the cells after five days in RPMI-1640 medium. 10X magnification.



Graph 1: Proliferation of cells of the parotid gland treated with minimal dosages and above the recommendations of the literature. The number of cells approximate times the power of 10^5 .

The results presented so far reflect only the effects of cell isolation and alpha-tocopherol as a non-harmful antioxidant to the cell culture studied in this research. The results below, in turn, express the data collected to observe the treatments used in the study.

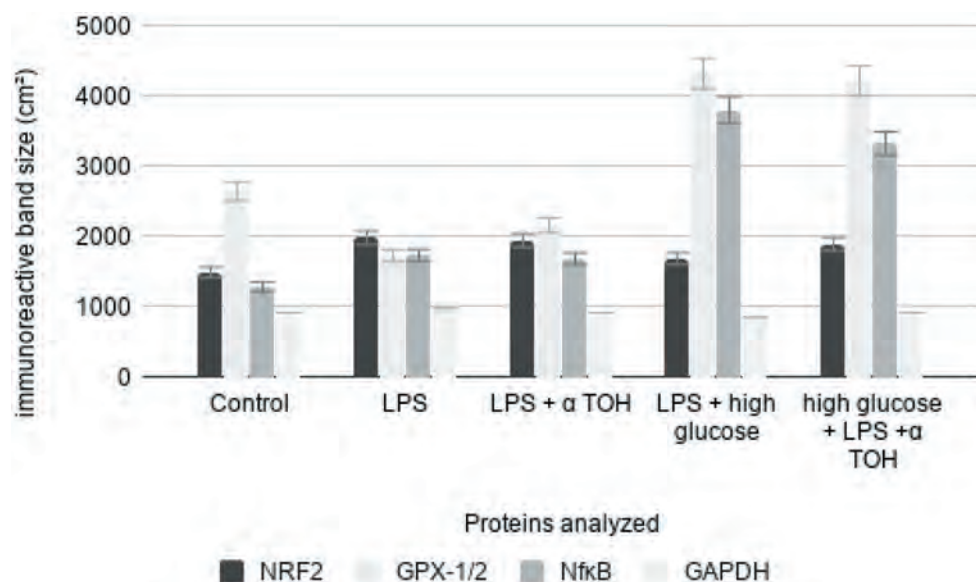
Discussion

DM is a chronic metabolic disease that affects the gland tissues involved in insulin secretion and glucose metabolism. One of the main symptoms related to diabetes is the presence of hyperglycemia, which is an important cause of a chronic inflammatory state and stimulates

reactive oxygen species, among other radicals, which are highly harmful to cellular structures [27,28].

Thus, the preservation of organelles and biomembranes of the cells affected by the harmful conditions generated by hyperglycemia, in addition to blocking the inflammatory condition, consists of an attempt to delay the harmful progress of this condition [29]. In this sense, alpha-tocopherol is an efficient complementary treatment in several experimental contexts [21-23, 30].

Wallert and collaborators [31] observed that alpha-tocopherol reduced the infiltration of monocytes and neutrophils in the cardiac tissue of C57BL/6 mice that suffered an acute



Graph 2: Immunoblotting of proteins involved in cellular metabolism as an antioxidant and inflammation. Nuclear factor erythroid 2-related factor 2 (NRF2) is short for type 2 nuclear erythroid factor protein. GPX-1/2 is short for type 1 and 2 glutathione peroxidase protein. NF-κB is short for nuclear factor-kappa B. GAPDH is the control protein for this type of test.

myocardial infarction. Besides, reductions in the levels of reactive oxygen species and peroxidated lipids were also observed. In the same perspective of improvement, Özgül and collaborators [32] found that the administration of 30 mg/kg intraperitoneally, via alpha-tocopherol diluted in saline to Sprague-Dawley animals with acute pancreatitis, attenuated the harmful effects of the disease and showed improvements in the levels of lipase and amylase expressed in blood plasma.

In the present study, the observed results corroborate those available in the literature [21-26, 30-32]. It was found that cell cultures that were in contact with the prescribed dosages of alpha-tocopherol (100 ng/ml) suffered less cell death than the groups that were exposed to harmful agents, LPS and hyperglycemia.

Another result of great importance was the increase in the protein glutathione peroxidase (GPX-1/2), available in intracellular medium and involved in antioxidant processes of conversion of hydrogen peroxide into water and oxygen, a process mediated in conjunction with the enzyme intracytoplasmic catalase, in groups submitted to stressors [33]. In this sense, the LPS group and hyperglycemia, group four, had the highest levels of this protein.

Corroborating this, the same group showed high levels of nuclear factor kappa B (NfκB) expressed by the immunoblotting assay. The NfκB is an important protein involved in the inflammatory process cycle that induces the transcription of the genes responsible for encoding the cytokines interleukin 1, interleukin 6, and the tumor necrosis factor in its most diverse isoforms [34]. This is because, once NfκB is expressed, a process that is mediated by the presence of stress, free radicals, ultraviolet radiation, and harmful microscopic agents, the ReLA p50 protein is stimulated, which can penetrate the cell nucleus and trigger the action of the RNA polymerase protein on the transcription of genes related to pro-inflammatory proteins [35]. The cellular effects observed after the installation of this complex system are associated with greater cell death and tissue necrosis, as well as decreased parenchyma and increased tissue stroma [36].

From the observed results, it appears that the cells of the parotid gland responded to the presence of inflammatory agents by increasing the intracellular antioxidant defense and by signaling local inflammation, a fact that in an organic perspective would trigger the advance of mononuclear phagocytes to the signaled places to fight the sources of inflammation [37].

Once the alpha-tocopherol was administered, according to the experiment carried out with group five, the expression of NfκB decreased, without affecting the reduction of GPX-1/2. It assumes that this behavior results from a joint action of antioxidant molecules, with alpha-tocopherol acting in an extracytoplasmic environment, preventing the stress of biomembranes, mainly cytoplasmic, and glutathione peroxidase acting inside the cell.

The importance of alpha-tocopherol is recognized as an antioxidant that acts in the cellular environment in an extracellular way [30]. Its main action as an antioxidant is to donate electrons to unpaired radicals and thereby stabilize them before they oxidize cell membranes [24]. Subsequently, once the alpha-tocopherol is oxidized, it is possible to reduce it again through the union with other tocopherols present in the extracellular medium or through the relationship with ascorbic acid [38].

In a study published by Ausili and collaborators [39], it was identified that alpha-tocopherol is closely related to the lipid portion of biomembranes so that variations in the electronic density profile of these cell structures are not identified. Still, it was observed by the authors that the alpha-tocopherol is located very close to the lipid-water interface, a fact that corroborates the observations made by other authors, such as Quin, Yu, and Yu, who observed that the alpha-tocopherol is located in this region of the biomembrane. but it oscillates horizontally along with the structure so that it is not restricted to a single area [40].

Thus, it appears that alpha-tocopherol showed antioxidant effects comparable to those already documented in the literature, however, the effects of this molecule were not known in detail under the conditions experienced in this study and the cells used.

Conclusion

Alpha-tocopherol showed antioxidant effects and attenuated the inflammatory progression by decreasing the expression of the nuclear factor kappa B (NfκB) protein in cells

of the parotid gland of mice submitted to high doses of glucose and lipopolysaccharides. Therefore, further studies are needed to understand the effects of this vitamin on animal and human conditions.

Conflict of Interest

The authors declare no conflict of interest.

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Original Research

The Effect of Aerobic Training and Supplementation of Curcumin on the Glycemic Markers and Level of Myeloperoxidase Enzyme of Overweight Women

Leila Farzad¹, Sepideh Dolati^{2*}, Alemeh Hariri Far³

¹ Department of Nutrition, Iran University of Medical Sciences, Tehran, Iran

² Department of Nutrition, ministry of health, Tehran, Iran

³ Department of Community Nutrition, Faculty of Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran

*Correspondence to: Sepideh Dolati, Department of Nutrition, Ministry of Health and Medical Education, District 2, Eyvanak Blvd of Industry Square, Tehran, 1467664961, Iran. Email: sd.dolati@gmail.com. Phone: +98 -81454977; Fax: + 98-81454357.

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Abstract

Background and Aims: Diabetes and cardiovascular disease are the most common diseases in the world. Diabetes has many complications and heart disease causes deaths worldwide. This study was aimed to investigate the effect of curcumin supplementation alone and with aerobic training on Body Mass Index (BMI), insulin resistance and myeloperoxidase (MPO) level in overweight women. **Material and Method:** In this randomized double-blind placebo-controlled trial, forty healthy sedentary overweight females aged 30–45 years and with a BMI 25–30 kg/m², were assigned to four groups of ten subjects each: “Curcumin supplementation (Cur)”, “placebo (Pla)”, “Cur+aerobic training (Tra)” and “Pla+Tra”. The aerobic training program comprised 3 days per week and Curcumin or placebo (500 mg/day) was administered in Cur and Cur+Tra groups in 8 weeks. Before and after intervention, BMI, HOMA-IR (Homeostatic Model Assessment-Insulin Resistance) and MPO enzyme were measured. ANOVA and ANCOVA was used to assess differences between the groups. **Results:** The within-group analysis showed no significant difference in BMI, HOMA-IR and MPO in four groups ($p > 0.05$). Aerobic training and curcumin supplementation had no significant effect on BMI, HOMA-IR, and MPO levels in overweight middle-aged women ($p > 0.05$). **Conclusions:** These findings suggest the combination of curcumin supplementation with aerobic training has no significant effect on BMI, HOMA-IR, and MPO levels in overweight middle-aged women. Further studies with different doses and longer interventions are needed to clarify the results.

Keywords: Aerobic Training, Curcumin, Diabetes mellitus, Peroxidase.

Background and Aims

In recent decades, due to inactive and sedentary lifestyles, cardiovascular disease (CVD) has increased [1, 2]. CVD is one of the most important causes of mortality, worldwide [3]. Although CVD mortality has reduced in high-income countries, it is rising in low and lower-middle-income countries [3].

The Nurses' Health Study (NHS) cohort study found that most women gain weight through middle adulthood. The women enrolled in the study were selected as a homogeneous population, predominantly caucasian, with high socioeconomic status and having a nurse occupation [4]. Other studies showed the prevalence of obesity among women more than in men, in developing countries [57]. This



condition has an important effect on health impairment, many diseases, and mortality [4, 8, 9]. Previous studies have shown the effect of overweight and obesity on insulin resistance index [10]. Insulin resistance has been associated with metabolic problems and some diseases [5, 11, 12]. The result of one study showed that aerobic training of eight weeks reduced insulin resistance in obese women [13]. Therefore, aerobic training is a good measure to control and improve the risk factors of insulin resistance such as diabetes and CVD [13].

Also, the results of some studies have shown prolonged or short-duration high intensity exercise causes increase of radical production, oxidative stress, and damage to body cells in active skeletal muscles [14]. Exercise-induced oxidative stress increases the production of free radicals. These free radicals are highly reactive and have short half-lives therefore, are difficult to measure [15]. As a result, the oxidative stress markers are used to measure oxidative stress. These markers indicate increase in free radicals [15]. One of these markers is myeloperoxidase (MPO). MPO is a myeloid-lineage restricted enzyme that is largely expressed by neutrophils and found in the azurophilic granules. According to the results of some studies, this marker is also produced in pathological conditions from monocytes and macrophages [16]. MPO has powerful antibacterial properties and defends humans against various pathogens by phagocytosis. MPO employs H_2O_2 to produce hypochlorous acid (HClO) and other reactive parts, which destroy pathogens during infections. In contrast, many studies have shown elevated blood MPO level is associated with inflammation, CVD, neurodegenerative, autoimmune, and cancer diseases [16–19]. In addition, some studies have also shown that intense physical activity can cause degranulation of neutrophils, leading to an increased blood MPO concentration. Exercise-induced muscle damage is associated with the activation of leukocytes and onsets an inflammatory response. Some studies have shown oxidative stress following intensive training [20].

In recent years, many researchers have paid attention to the use of spices on the prevention and treatment of various diseases [21].

Curcuminoids are one of the most important anti-inflammatory agents in nature. It is found in turmeric rhizomes, of *Curcuma longa* L. [21, 22]. Yellow pigments of curcuminoides are its main active components [21]. It has many therapeutic effects in various diseases including chronic inflammatory diseases, obesity, depression, and fatigue [21]. In metabolic syndrome, curcumin can decrease leptin concentrations [23]. It is a bio-active polyphenol found in turmeric rhizomes, of *Curcuma longa* L., (Zingiberaceae), and has multiple pharmacological actions [21, 22]. Yellow pigments of curcuminoides including 3–5% of turmeric composed of curcumin, demethoxy curcumin, and bisdemethoxy curcumin are main active components [21]. There is an evidence that curcumin is an anti-inflammatory agent, so, it has many therapeutic effects in various diseases including chronic inflammatory diseases of Alzheimer's, Parkinson's, multiple sclerosis, brain injuries, CVD, types of cancer, allergies, asthma, arthritis, obesity, depression, and fatigue [21]. In metabolic syndrome, curcumin can decrease Body Mass Index (BMI), weight, waist circumference and leptin concentrations [23].

A study showed that regular consumption of curcumin with regular aerobic training improves endothelial function significantly [24]. This research has shown 150 mg curcumin supplementation, daily, with aerobic training, for eight weeks improves endothelial function in healthy postmenopausal women [24]. In a study of the effect of endurance training with curcumin intake on the hemodynamics of the central arteries of healthy postmenopausal women, it was concluded that daily intake of 150 mg curcumin in combination with aerobic training, three to six sessions per week for eight weeks, reduced BMI significantly [25]. Evaluation of the effect of curcumin on rheumatoid arthritis patients showed that daily consumption of 500 mg curcumin led to inflammatory index of C-reactive protein [26].

Most studies have investigated the effects of different training or curcumin supplementation, separately on myeloperoxidase levels. No study has found a combination of them in overweight middle-aged women. Therefore, this study aims to answer the question whether

aerobic training and curcumin supplementation have an effect on myeloperoxidase enzyme levels and body composition in overweight middle-aged women.

Material and Method

Study design and patients

Forty healthy sedentary women (less than 30 minutes a day of moderate intensity physical activity for three days a week [27], overweight, and aged 30–45 years) participated in this study who were referred to the comprehensive health center or other health centers of Tehran University of Medical Sciences, located in Yaft-Abad district. Participants were recruited only if they met the following criteria: $24.9 < \text{BMI} < 29.9 \text{ Kg/m}^2$, no allergy to curcumin, no pregnancy, non-smoking, no tobacco and hookah consumption, no following a special diet, no underlying or infectious disease, no known history of high blood lipid without medication, cardiac failure, taking anti-inflammatory drugs, severe endocrine or metabolic disorders, hospitalization for any reason, and no intake of dietary supplements and herbal tea. All volunteers completed a self-administered questionnaire on demographic, physical activity, exercise, dietary intake, lifestyle information, and health history prior to the study. While none of the study participants were trained in physical activity or aerobic exercises of any form and type in the past 6 months.

In this method a simple randomizing model like drawing of lots was employed. In this case, the sample was categorized in control and intervention group according to the result of lots. The Fleiss equation for sample volume estimation was used in this survey in order to approximate the volume of sample; in which the power of experiment, alpha of equation and the average alteration were 0.8, 0.05 and 5, respectively. According to the conducted estimation, the sample size was calculated 8.81. To be more cautious, among the overweight volunteer women ten persons were chosen as the experimental group.

A double-blind, placebo-controlled, clinical trial was used and performed from

January to March 2018. The participants randomly underwent, in one of laboratory-based trials, in four randomized orders by a list prepared with a random number generator in four groups of ten: (1) placebo supplementation (Pla), (2) curcumin supplementation (Cur), (3) curcumin supplementation besides aerobic training (Cur+Tra), and (4) placebo supplementation besides aerobic training (Pla+Tra). The Cur and Tra groups, took two curcumin capsules in a day (500 mg/day) and the Pla and Pla+Tra groups received a placebo every day, for eight weeks.

This study was approved by the Ethical Committee at the Azad University - Tehran Branch and registered in the Iranian Registry of Clinical Trials (NO: IR.SSRI.REC.I397.209). Written informed consent was obtained from all participants at the beginning of the study.

Laboratory, anthropometric and clinical data collection

Height and weight were measured using portable measuring rod and electronic digital scales (Beurer BG55 Digital Scale, RU), accurate to 0.1 kg, respectively. Anthropometric indexes were measured by one observer. Subjects were in light clothing and no shoes and height was measured in the standing position. BMI was calculated as body weight (kg) divided by squared height (m^2). Then, subjects were categorized as underweight, normal weight, overweight, or obese according to the international classification used by the WHO [28].

Venous blood samples were taken from the left vein in the sitting position in the early morning after overnight fasting. For measuring serum blood markers, samples were centrifuged. The glucose oxidase method (Using the Spanish Biosystems glucose measurement kit) Fasting blood insulin levels were estimated by Enzyme-Linked Fluorescence Assay (ELFA) method (TOSOH kit, Japan) in all the study participants. The insulin resistance index was calculated by the following formulas: $\text{HOMA-IR} = \text{Fast insulin } (\mu\text{U/ml}) \times \text{Fast blood glucose (mmol/L)} / 22.5$. Plasma MPO concentration was determined by immunoassay using a commercial kit

(Immunology Consultants, Laboratory, Inc., Newberg, USA). Intra-assay and assay-inter values were 2.8% and 6.5%, respectively. The separation and recovery limit of this assay was 0.121 ng/ml and 86.7%, respectively.

Turmeric powder was provided by the reputable brand of Golha Company (Iran) in supervision of Analysis center of the institute of medicinal plants. 5000 g of the powder was poured into 5000 ml 80°C alcohol. The Percolation method was used for the extraction of alcohol. After 24 hours, the contents were smoothed using a Buchner funnel and ordinary filter paper. The extraction efficiency was 10%. The powder was dispersed in a suitable solvent (80% ethanol) to prepare the drug form. After expanding, breadcrumbs were used as expanders and fillers to achieve proper fluidity. Based on previous clinical studies, an effective dose of 250 mg of the ethanolic extract is estimated to be 80%. Total curcuminoids have been estimated at 75–85% in previous studies [29]. Therefore, 250 mg capsules (00 short stays manufactured by Iran Gelatin Company) were prepared with a semi-automatic encapsulating machine. The Pla and Pla+Tra groups received two placebo capsules per day, which were similar in shape and color and made of an equivalent dose of starch. The study duration was eight weeks and all subjects were instructed not to alter their dietary habits during this period.

Participants in the Cur+Tra and Pla+Tra groups underwent aerobic training (three days per week for eight weeks) in a gym. The aerobic capacity of the subjects was examined by a 6-minute Rock-Port test. The maximum heart rate for each person was estimated as equal to $208 - 0.7 \times (\text{age})$ [30]. In the training session, first, the subjects did warm up stretching exercises for 6 minutes, then were running continuously on the treadmill, at a speed of 7 km/h, by heart rate 50–80% of maximum, for 20 minutes. Their heart rate was monitored by a polarimeter (Polar: Finland, within a 5% error rate of a heartbeat) during running, to keep it in the specified intensity. This intensity was individualized. Every person had certain intensity. The Youngers run with higher intensity and the older ones run with lower intensity. In each of the next sessions, 90 seconds were added to the running time. The training

intensity was controlled using a belt heart rate sensor (polar beat). Subjects in the Cur and Pla groups were instructed not to change their level of physical activity.

Statistical analysis

The mean $\pm$ SD were used to describe the results. The Kolmogorov-Smirnov test was used to determine the distribution of data. Covariance test was used to determine the effectiveness of independent variables on the dependent variables. 95% confidence level was considered and p-value was considered significant at 0.05 level. Statistical analysis was performed using SPSS 22 software (SPSS Inc., USA).

Results

The mean $\pm$ SD of age, weight, height, BMI, HOMA-IR, and MPO of subjects in the pre-test are presents in Table 1, and Pre-test and post-test values of the studied variables in the subjects have been shown in table 2. The Kolmogorov-Smirnov test was used to check for normality of distribution of all variables. The distribution of these variables did not differ significantly from normal.

Baseline characteristics of the participants in different groups

The results of ANOVA test on BMI, HOMA-IR, and MPO of subjects in pre-test are given in Table 3. As seen, the results are not significant for any of the variables ($P > 0.05$) in baseline between groups.

The effects of aerobic training, curcumin, and their interaction on variables

The results of analysis of covariance to determine the effect of aerobic training and curcumin supplementation on MPO enzyme levels, HOMA-IR, and BMI in overweight middle-aged women are presented in the table 4.

Table 1: The central indices and distribution of individuals in the pre-test^a

Variables	Groups			
	Cur	Pla	Cur+Tra	Pla+Tra
Age, y	38.9±5.40	40.6±3.71	35.8±3.22	38.2±5.67
Height, m	159.09±4.64	162.3±4.91	162.44±4.36	159.58±6.98
Weight, kg	67.31±6.54	71.31±5.73	74.45±6.63	70.37±9.23
BMI, kg/m ²	26.96±1.90	27.21±1.43	28.18±1.70	27.52±1.70
HOMA-IR	2.00±1.22	1.72±0.91	1.78±0.64	2.12±0.82
MPO, ng/ml	9.33±2.73	8.78±3.13	9.86±3.81	9.15±3.6

Abbreviations: BMI, body mass index; MPO, myeloperoxidase; Cur, Curcumin; Pla, Placebo; Cur+Tra, Curcumin+Training; Pla+Tra, Placebo+Training; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance;

^a Values are expressed as mean±SD;

Table 2: Pre-test and post-test values of the studied variables in the subjects^a

Variables	Groups							
	Cur		Pla		Cur+Tra		Pla+Tra	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
BMI, kg/m ²	26.96±1.9	26.51±1.8	27.21±1.4	26.80±1.5	28.18±1.7	27.94±1.5	27.52±1.7	27.06±1.8
HOMA-IR	2.00±1.2	1.70±0.6	1.72±0.9	2.04±1.1	1.78±0.6	1.62±0.5	2.12±0.8	1.73±0.4
MPO, ng/ml	9.33±2.7	10.16±2.7	8.78±3.1	8.75±3.2	9.86±3.8	11.09±4.1	9.15±3.6	10.24±3.8

Abbreviations: BMI, body mass index; MPO, myeloperoxidase; Cur, Curcumin; Pla, Placebo; Cur+Tra, Curcumin+Training; Pla+Tra, Placebo+Training; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance;

^a Values are expressed as mean±SD;

Table 3: ANOVA test results on BMI, HOMA-IR, and MPO of subjects in pre-test

variable		sum of squares	Df	average of squares	F	P Value ^a
BMI, kg/m ²	Inter-group	8.397	3	2.799	1.073	0.373
	Within-group	93.916	36	2.609		
	Total	102.313	39			
HOMA-IR	Inter-group	1.71	3	0.57	1.454	0.244
	Within-group	5.53	36	5.53		
	Total	7.24	39			
MPO, ng/ml	Inter-group	6.058	3	2.019	0.180	0.909
	Within-group	403.846	36	11.218		
	Total	409.904	39			

Abbreviations: BMI, body mass index; MPO, myeloperoxidase; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance;

^aP values were considered significant, statistically, less than 0.05.

Table 4: ANCOVA results on the effect of intervention on studied variables in subjects

variable	factor	sum of squares	Df	average of squares	F	Sig. ^a	Effect Size (μ)
BMI, kg/m ²	Pre-test	4.11	1	4.11	1.53	0.220	0.407
	group	15.48	3	5.16	1.92	0.134	0.057
HOMA-IR	Pre-test	5.53	1	5.53	14.08	0.001	0.287
	group	1.71	3	1.71	1.454	0.244	0.111
MPO, ng/ml	Pre-test	2.07	1	2.07	0.194	0.661	0.337
	group	44.61	3	14.87	1.38	0.254	0.187

Abbreviations: BMI, body mass index; MPO, myeloperoxidase; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance;

^aP values were considered significant, statistically, less than 0.05

Based on the results in Table 4, it was revealed that pre-test did not have any significant effect on BMI changes in overweight middle-aged women. That is, after eliminating the pre-test effect, there was no significant difference between the mean BMI in the groups. Accordingly, it was found that after eliminating the pre-test effect or the same covariance factor, there was no difference in mean BMI. Therefore, aerobic training and curcumin supplementation and the combination of these two interventions have no effect on overweight middle-aged women's BMI. Also, by removing the effect of insulin resistance values in the pre-test as the covariate variable, the main effect of group variables on the post-test scores was not significant ($F=1.454$, $P=0.244$, $\mu=0.111$). In other words, there was no significant relationship between aerobic training and curcumin supplementation in subjects and their mean HOMA-IR ($P>0.05$) between groups. The results of covariance analysis showed that pre-test had no significant effect on MPO enzyme level changes in samples. Accordingly, it was found that after eliminating the pre-test effect or the same covariance agent, there was no difference between the mean levels of MPO enzyme ($F=0.194$, $P=0.661$, $\mu=0.337$). Thus, curcumin supplementation, alone, does not affect the level of MPO in overweight middle-aged women.

Discussion

In the current study, the influence of aerobic training and curcumin supplementation

on myeloperoxidase enzyme levels, BMI, and HOMA-IR in overweight middle-aged women was investigated. Overall, the results of this study showed that aerobic training and curcumin supplementation had no significant effect on myeloperoxidase enzyme levels, BMI, and HOMA-IR in overweight middle-aged women.

According to the findings of this study, aerobic training and supplementation of curcumin had no significant effect on myeloperoxidase in overweight middle-aged women. The results of this hypothesis are in line with the research of Dastani et al. [31], and Duzova et al [32]. In a study of the effect of a selected stationary training course on plasma myeloperoxidase in inactive women by Dastani et al. it was shown that intra-group changes in serum myeloperoxidase levels were not significant in any of the experimental and control groups. Also, inter-group changes were not statistically significant. Duzova et al. investigated two methods of continuous moderate training (60 minutes) and intense running (20 minutes) on treadmill in rats for five weeks. Results of the study did not report a significant change in plasma and muscle MPO in rats in any of the groups [32], which is consistent with the results of the present study. In contrast, Morozov et al., when performed very intensive training in rats with a program including repetitive swimming by adding rats, concluded that very intensive training increased the MPO level of muscle immediately and 24 h after training [33]. It seems that these results are inconsistent with the results of the present

study, and it may be due to the type of training and the duration of activity in this study. Also, the results of this study showed that 8 weeks of curcumin supplementation and aerobic exercise decreased the insulin resistance index and body mass index after the intervention but this decrease was not significant ($P > 0.05$). A study by Maithilikarpagaselvi et al. in mice showed that curcumin consumption after 10 weeks significantly decreased the insulin resistance index [34]. This result is similar to results of Shao et al. [35] and Teich et al. [36] studies. In Shao study, the duration of curcumin intake was 28 weeks. The studies of Bai [37] and Azza [38] have shown similar findings. But, none of the studies reviewed showed inconsistent results with the above findings.

On the other hand, various studies have shown the effect of aerobic training on insulin resistance index. A study by Nezamdoust et al. on type 2 diabetic patients shows that 12 weeks of aerobic training for three sessions per week has a significant relation with insulin resistance index.

This study was a short-term trial that it is unclear whether curcumin supplementation will have significant effects on BMI, HOMA-IR, or MPO in the longer term or not. This is a limitation of this study. Also, the impact of different levels of doses was not investigated in the current research.

It is recommended to investigate the effect of strength training (or interval training) and curcumin supplementation on myeloperoxidase enzyme levels, BMI, and HOMA-IR in overweight middle-aged women.

Conclusions

The results of this study conducted showed that independent variables (aerobic training and curcumin supplementation) had no significant effect on the dependent variables (MPO enzyme and BMI). The results of this study were compared with previous research. The findings of this study appear to be inconsistent with previous studies. Some similar studies showed that these independent variables had a significant effect and others showed no significant effect. However, different reasons can be considered such as age, animal

subjects to humans, intensity of training, amount of supplementation and others. Therefore, it is suggested that these two variables should not be used to control MPO and BMI.

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Conflict of Interest

The author declares no conflict of interest.

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Case Report

Digital and Conventional Approach for the Treatment of Severe Anterior Dental Wear in a Type 2 Diabetes Patient

Raul Rotar, Codruta Ille*, Anca Jivanescu

Department of Prosthodontics, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania

*Correspondence to: Codruta Ille, Department of Prosthodontics, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania, E-mail: codrutaille@yahoo.com, Phone: +40746382959

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Abstract

Introduction: Nowadays, due to a multitude of physical and psychological factors, more and more patients suffer from dental wear. The overlapping of metabolic disorders often leads to oral effects that generate major functional and esthetic inconveniences, which can be challenging even for experienced clinicians. **Case Report:** We present a senior female patient with type 2 diabetes mellitus and severe anterior dental wear associated with poor oral hygiene, periodontal disease, and tooth loss in the posterior dental arches. After general health evaluation, surgical, periodontal and endodontic treatments were conducted, followed by a combined prosthodontic rehabilitation treatment plan which involved the conventional and digital fabrication of dental restorations. **Conclusions:** Clinical results showed that the esthetic and functional outcome of the treatment was achieved even when combining the two methods (digital and conventional) of fabricating dental restorations and the so-called sixth complication of diabetes, the periodontal disease was diminished, due to optimal hygiene allowed by the design of the restorations.

Keywords: Type 2 diabetes, severe dental wear, CAD/CAM restorations.

Introduction

Diabetes has reached the status of being one of the most prevalent noncommunicable diseases of senior people [1]. As a result, the health concerns are serious due to the large number of older patients that require dental treatments that, among other associated health problems, present metabolic diseases. Depending on the type of diabetes, the dental approach needs to undergo specific steps. The prevalence of type 1 diabetes is relatively low compared to the total number of diabetic patients and represents approximately 5% [2]. However, it is estimated that worldwide, 1 out of 11 people have a metabolic disease, out of which 90% fall in the type 2 diabetes (T2D) category [3]. The leading causes of a drastic increase in the T2D cases range from bad eating habits, lack of physical activity, and population aging [4, 5]. One of the main concerns regarding T2D

is represented by the fact that the disease occurrence precedes the diagnosis by several years [6]. This leads to an increased risk of developing complications that impact both the overall health and dental status [7]. T2D reduces the life expectancy of patients and, in association with other risk factors such as genetic predisposition and high body mass index, leads to millions of deaths annually [8, 9].

Another common characteristic of the senior population is represented by the wear of the teeth that are still present in the oral cavity. The progression of the wear is usually constant throughout life and influences the ability to efficiently chew the food [10, 11]. The most common factors that lead to tooth wear are represented by the mechanic grinding of opposing teeth structures (attrition), wear due to contact with structures other than teeth (abrasion), and loss of tooth structure due to chemical agents (erosion) [12, 13].



Although tooth wear starts from an early age, it usually becomes evident in the later stages of life, and in some cases, the development of the process is so advanced that it drastically diminishes the function and esthetics of the teeth [14-16]. The overlapping of metabolic diseases such as T2D, through its influence on teeth supporting structures, may lead to severe tooth mobility, and without a systematic periodontal and prosthodontic treatment, the outcomes will lead to the loss of the afflicted teeth [17].

Prosthetic rehabilitation of patients with T2D and severe anterior tooth wear can be a challenging situation, and this case report illustrates the functional and esthetic outcome achieved with a combination of digital and conventional methods for dental prostheses fabrication.

Case report

A 63 years old female patient with T2D was addressed to the Department of Prosthodontics of the Faculty of Dentistry from Timisoara, and the main complaint was major esthetic problems in the anterior arch as well as functional problems in the lateral areas.

All papers regarding personal data protection, informed consent for the treatment, and the acceptance for digital photography were signed by the patient, and the medical and dental history was investigated. The anamnesis revealed a T2D metabolic disorder diagnosed at the age of 59, which has never been under rigorous control (HbA1c=7.1%). The main identified cause for the increased glycemic index was the increased daily sugar intake. The physical examination showed a height of 161 cm and a bodyweight of 72 kg with a BMI of 27.8.

During the first appointment, the clinical examination was performed. The extraoral examination showed no evident asymmetries, adequate muscle coordination, medium-high smile line, and accentuated perioral folds due to the decreased vertical dimension of occlusion. There was no visibility of upper anterior teeth in the rest position due to excessive wear (Figure 1). No pain was reported in the temporomandibular joint, the lymph nodes were not palpable, and the



Figure 1: At presentation, in the rest position, there is no visibility of front upper teeth.

mouth opening amplitude was within normal limits.

The intraoral examination revealed gingivitis in the area of mandibular anterior teeth, fixed dental prostheses in the posterior mandible, and wear of all upper anterior teeth (Figure 2).

The main objectives of the treatment were the prosthodontic rehabilitation of the function and the esthetics and also the control of periodontal disease, facilitated through the design of the final restorations.

The first step included a set of preliminary alginate impressions which were poured and mounted in a semi-adjustable articulator (Artex, AmannGirrbach). The occlusal analysis showed increased tooth wear in the upper frontal arch associated with occlusal interferences. A maxillary diagnostic wax-up was manufactured in the dental laboratory with an increased vertical dimension of occlusion (VDO) of 3 mm. Based on this wax-up, direct provisional restorations were



Figure 2: Excessive wear of upper front teeth.

manufactured (Luxatemp, DMG) and assessed for esthetics and acceptance from the patient.

Upon the patient's approval, the second phase of the treatment was initiated, which involved the removal of the old fixed dental prostheses and the periodontal and endodontic treatment of the remaining abutments.

Next, another set of direct provisional restorations was made and set in place with a temporary luting cement for three weeks, allowing the patient to adjust with the increase in the VDO.

A combined conventional and digital treatment plan was chosen. The reason behind the treatment plan was to offer the best possible outcome with the limited financial resources provided by the patient.

For the lateral arches, metal-ceramic fixed partial restorations were placed on the remaining abutments. Due to the old restorations' previous design, the abutments were prepared with a 'no shoulder' finish line. Then, the design of the fixed partial dentures followed the design of the provisional restorations. The final restorations were adjusted and luted with temporary cement. During these stages, the provisional restorations were kept in place on the upper frontal arch.

Next, for the upper front teeth with wear, a minimally invasive approach was implemented, with four rounded shoulder crown preparations on the incisors and a veneer preparation on the canine. Also, the minimal reduction of the teeth surfaces avoided the endodontic treatment (Figure 3).

The preparations were scanned with the intraoral scanner (PlanScan, Planmeca), obtaining the digital model (Figure 4). All the digital design steps were followed, including scanning

the opposing arch and occlusion, placement of the finish lines, and designing the final ceramic restorations (Figure 5).

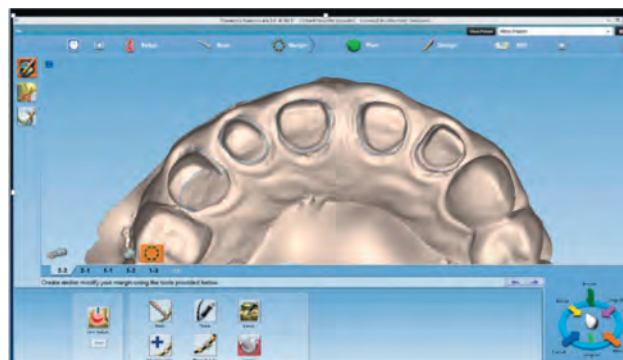


Figure 4: Digital model obtained after intraoral scanning with PlanScan (Planmeca).

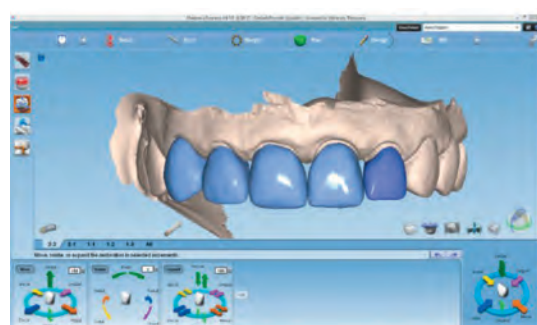


Figure 5: Digital design for one veneer and 4 crowns obtained with PlanCAD Easy (Planmeca).



Figure 3: Conservative preparation of the front teeth.

After in-office milling (with Planmill 40, Planmeca) of leucitic ceramic blocks (Empress CAD Multi ceramic blocks) (Figure 6), an initial clinical trial of the restorations was performed, and some minor adjustments were made and then sent for glazing to a dental technician.

Adhesive cementation protocol was conducted with light-curing resin cement (Variolink Esthetic LC, Ivoclar, Vivadent), and the final result brought satisfaction to the clinician's team and also to the patient.

Discussion

In general, the patients who suffer from a metabolic disease such as T2D can be treated in the dental office without significant complications. The key to a successful treatment is



Figure 6: Empress CAD multi block, after removal from the milling machine.

the constant check-up of the glycemic values combined with the appropriate medication, the cooperation, and involvement of the patient being of the utmost importance to keep the disease under control [5].

Prosthodontic treatment is not necessary in all cases of tooth wear. Factors related to the severity of tooth wear relative to the age of the patient, the etiology, the symptoms, the progression rate, and the patient's expectations should be taken into consideration in the attempt to decide the treatment [18-20]. Metal-ceramic fixed dental prostheses were selected for the lateral area since they seem to be the safest choice in cases of high load conditions. Individual ceramic restorations were selected in the anterior arch because torque forces are minimized. Even if cementation failure occurs, detection and repair would be easier in crowns than in multiple unit bridges, where a single abutment de-cementation is challenging to be detected. Moreover, all-ceramic restorations provide superior esthetic results and sufficient strength as long as the occlusal contacts are adequately balanced.

Conclusions

Proper occlusal adjustment of the metal-ceramic restorations, a protective stabilization splint, and frequent recall and maintenance visits minimize the incidence of clinical complications. This is even more imperative in the cases of patients who have an overlapped metabolic disease due to its significant influence



Figure 7: Empress CAD multi ceramic restorations after adhesive cementation on upper front teeth.

on teeth supporting structures. The involvement of the patient in maintaining oral hygiene and also the glycemic values within normal limits is the only way for having long-lasting restorations.

Conflict of Interest

The authors declare no conflict of interest.

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Case Report

Diagnosis and Management of Pheochromocytoma in a Resource-Challenged Setting: A Case Report

Esohe Odotayo^{1,2}, Ijeoma Onyinye Ohuche^{1,2*}, Muhammad Faruk Bashir^{1,2}, Abel Olumuyiwa Alonge^{1,2}, Nuraddeen Ibrahim^{1,2}, Laila Matogah Adutwum^{1,2}, Adele Bodieu Chetcha^{1,2}, Mawse Amouzouvi-Sadji^{1,2}, Elizabeth Eberechi Oyenusi^{2,3}, Olumide Abiodun Elebute^{2,4}, Felix Makinde Alakaloko², Adesoji Oludotun Ademuyiwa^{2,4}, Abiola Olufunmilayo Oduwole^{2,3}, Ibrionke Desalu^{2,5}

¹ Paediatric Endocrinology Training Centre for West Africa, Lagos Nigeria

² Lagos University Teaching Hospital, Lagos, Nigeria

³ Department of Paediatrics, College of Medicine, University of Lagos, Nigeria

⁴ Department of Surgery, Paediatric Surgery Unit, College of Medicine, University of Lagos, Nigeria

⁵ Department of Anaesthesia, College of Medicine, University of Lagos, Nigeria

*Correspondence to: Ijeoma Onyinye Ohuche, Paediatric Endocrinology Training Centre for West Africa, Lagos Nigeria.

E-mail: ijeomaohuche@gmail.com. Phone: +234 806 763 8748

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Abstract

Phaeochromocytoma (PCC) is a rare catecholamine-secreting tumor derived from the sympathetic nervous system's chromaffin cells. Pheochromocytomas are exceptionally rare in children and are responsible for about 1% of pediatric hypertensive patients [1]. They arise most commonly in the adrenal glands (approximately 85% of tumors) [1] but may also arise from chromaffin cells of the sympathetic nervous system, known as paragangliomas (PGLs). The tumors are more common in boys than girls, with a 2:1 ratio of boys to girls in pediatric PCC/PGL cohorts [2]. PCC/PGL often occur as sporadic tumors, but may sometimes occur as part of hereditary tumor syndromes, mainly Von Hippel Lindau (VHL) disease and multiple endocrine neoplasia (MEN) type 2A and 2B [1]. About 50% of cases are missed and discovered at autopsy because the diagnosis was not considered at presentation [3]. We report this case of phaeochromocytoma to draw the attention of clinicians in our environment to this unusual cause of pediatric hypertension and the need for a thorough investigation of the hypertension etiology of in children. We also underline the fact that this feared tumor can be successfully managed even in areas with limited resources, such as ours. Finally, we encourage physicians to measure blood pressure of children presenting to their clinics.

Keywords: Phaeochromocytoma, pediatric, hypertension.

Case Report

A 14-year old girl presented to the pediatric clinic with complaints of recurrent headaches, visual disturbances, and an incidental finding of elevated blood pressure (BP), during a home-measurement of blood pressure by her mother, who is a nurse. She first presented to a pharmacy shop where anti-hypertensives were given to her. However, her blood pressure remained high. She

subsequently developed palpitations and excessive sweating, necessitating admission to the pediatric cardiology clinic where examination findings included elevated blood pressure, ranging from 150/120 to 220/150mmHg at repeat measurements, an irregular heartbeat, and a displaced apex beat at the sixth left intercostal space, at the mid-clavicular line. Following an assessment of hypertensive heart disease (secondary hypertension), a series of investigations were requested.



Chest X-ray, electrocardiography (ECG), serum electrolytes and thyroid function tests were normal. Two-dimensional echocardiography showed a structurally normal heart with mid-left ventricular hypertrophy but good biventricular contractility. The ophthalmological assessment was normal, and cranial computed tomography (CT) showed no abnormality. The abdominopelvic ultrasound scan showed a left extrarenal pelvis and a right aortocaval mass, which was identified by an abdominal CT scan as a right adrenal pheochromocytoma. Urinary normetanephrine was markedly elevated: 46642 nmol/24h (450-1709 nmol/24h), with a normetanephrine-to-creatinine ratio of 5481 (21-308). However, urinary metanephrine was within normal limits: 366 (100-1262 nmol/24h), with a urine metanephrine-to-creatinine ratio of 43 (26-188).

She was managed with anti-hypertensives—a combination of an alpha-blocker (prazosin) and a beta-blocker (propranolol), which was started two days after the commencement of the alpha blockade. She subsequently had near-total adrenalectomy two months after the initial presentation. A variegated firm to hard, right suprarenal gland tumor was found intra-operatively, adherent to the adventitia of the posterior wall of the inferior vena cava (IVC), and displacing it anteromedially. The mass was free from other surrounding structures. The IVC, right renal, and supra-renal veins were distended. Intraoperatively, she had blood pressure surges and dysrhythmias; hypertension was controlled using magnesium sulfate infusions and labetalol. The diagnosis of pheochromocytoma was confirmed by histology of the sample. Following surgery, all symptoms resolved over ten weeks. Biochemical markers have all returned to normal reference ranges and the patient is stable on follow-up. Other family members are yet to be screened due to financial constraints.

Discussion

This case highlights the diagnosis of a rare adrenal tumor, pheochromocytoma in a pediatric patient. This was an incidental finding, following a thorough evaluation of an

adolescent female with an incidental finding of hypertension after monitoring blood pressure at home.

Pheochromocytomas are rare neuroendocrine tumors, with an incidence of 1 per 100,000 population reported in the United States, with pediatric cases accounting for 10 to 20% of all cases in the US [4]. Some reviews of pediatric cases have shown that pediatric pheochromocytomas account for 8.86% of all cases of PCC/PGL [2]. Besides, they account for 1.1 to 11% of adrenal masses found incidentally [5].

Cases are more common in males than females, with a male-to-female ratio of 2:1 [6]. Incidence peaks between 9 and 12 years of age [6], with a median diagnosis age of 13 years [2].

Our patient presented with all of the four characteristic features of the tumor: headaches, palpitations, excessive sweating, and severe hypertension [4]. Hypertension from pheochromocytomas accounts for 0.5 to 2.5% of all pediatric hypertension [7] and has been shown to be present in 80-90% of children at diagnosis. Some studies have demonstrated that sustained hypertension (rather than paroxysmal), is seen in up to 90% of pediatric patients with pheochromocytoma [8]. The diagnosis is, nevertheless, an important cause of correctable hypertension in children [5]. Headaches are the most frequent symptoms in children, and are present in about 75% of patients, while palpitations are present in about 64% of cases [4].

Diagnosis of this rare and extremely perilous tumor requires a high index of suspicion since up to half of the cases are discovered at post-mortem examinations. Therefore, the diagnosis should be considered in any patient presenting with symptoms of excessive catecholamine secretion such as hypertension, palpitations (with tachycardia or, less frequently, reflex bradycardia), and excessive and inappropriate diaphoresis. Other less common symptoms include orthostatic hypotension, excessive anxiety, paleness of the face and upper body, tremors, heat intolerance, and, rarely, seizures [3]. Unfortunately, in our environment with high medical costs and lack of health insurance, presentation to a pharmacy with the purchase of over-the-counter medications is usually the

first line of treatment of symptoms for many patients, resulting in late presentation to the hospital.

Measurement of 24-hour urinary metanephrines (catecholamine metabolites) is recommended for the diagnosis of pheochromocytoma, with higher sensitivity for normetanephrine (87.3%) compared to metanephrine (56.9%) [10], as shown in our patient. The specificity of both tests is 95%. Measurements of plasma and 24-hour urinary catecholamines (epinephrine, norepinephrine, dopamine) and urinary vanillylmandelic acid (VMA) are no longer used, owing to the lower sensitivity and specificity of these tests. The elevation of catecholamine metabolites four times the upper limit of normal is highly suggestive of a tumor, and our patient had a urinary normetanephrine level that was 27 times the upper limit of normal.

The gold standard for diagnosis is the measurement of plasma or 24-hour fractionated urinary metanephrines with imaging studies performed for the localization, identification of metastatic lesions, and surgical resection [7]. There are a few laboratories in our environment able to measure fractionated catecholamine metabolites, thus limiting the accuracy of diagnosis in many cases. However, efforts should be made to contact laboratories with this competency, if the patient can afford it.

Ninety-five percent of adrenal tumors measuring 1 cm or larger, and 90% of pelvic and abdominal tumors larger than 2 cm are identified by computed tomography (CT) [3]. CT and MRI of the abdomen and pelvis remain the imaging modality of choice in pediatrics, with a sensitivity of 90-100% for both tests, and specificity of 70-80% [5]. MRI, however, has the advantage of a better sensitivity for extra-adrenal tumors, and no radiation exposure [5]. Nonetheless, this imaging modality is less affordable and accessible in our environment, and therefore, computed tomography is more commonly used for diagnosis, as seen in our index case.

Owing to financial challenges in our environment, where health insurance is not widely available, diagnosis in the index patient was first made by abdominal ultrasonography (which has a sensitivity of 89%) before computed tomography

was done to confirm ultrasound findings. This was further confirmed by 24-hour fractionated urinary metanephrine values and post-operatively by histological findings. In all, the lack of adequate finances considerably prolongs intervention time for patients in our environment, as shown by the 2-month duration from presentation to intervention in the presented case. Therefore, it is necessary to reiterate the need for functional health-insurance systems to be made operational in third-world countries, particularly in Sub-Saharan Africa. This would improve management outcomes for the vast majority of children living in these environments. Additionally, there is an urgent need for the subsidized care of the ever-growing number of children with cancers in our environment.

Management of pheochromocytoma is multidisciplinary, involving the pediatric endocrinologists, pediatric surgeons, anesthetists, and geneticists. Except for the geneticist, these subspecialties are readily available in most tertiary institutions in our environment. Pheochromocytomas are managed by complete surgical resection, which carries the risk of hypertensive crisis intraoperatively. The occurrence of this feared complication has been greatly reduced in recent times by the preoperative use of the alpha- and beta-blockade [8]. As presented in the index report, alpha blockade usually precedes beta-blockade, never vice versa, to prevent unopposed alpha-receptor stimulation, which would lead to a severe hypertensive crisis [11]. Malignant cases are further treated with chemotherapy, with a 5-year survival rate ranging from 78% to 95% [8]. Recurrences are frequent, especially in patients with germline mutations, and may occur as early as one-year post-surgery, or as late as 35 years later [8]. In view of this, long-term follow-up of patients is necessary; yearly measurements of urinary/plasma metanephrines and imaging studies should be carried out at regular intervals.

Conclusion

Endocrine hypertension in general, and specifically pheochromocytoma, should be considered in the differential diagnosis of children

presenting with severe hypertension, especially if accompanied by the triad of headaches, palpitations, and excessive sweating. This is important, especially in an environment like ours, where pediatric endocrine disorders are mostly undiagnosed.

Proper management by a multidisciplinary team is associated with good outcomes, especially in children with benign tumors.

Additionally, a functional health-insurance system in developing countries would improve the treatment outcomes for children who may present with the same or similar tumors, leading to improved survival of these children.

Conflict of Interest

The authors declare no conflict of interest.

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Review

The Handheld Infrared Thermometry in the Diabetic Foot – Useful but Debatable Technique

Elena-Rodica Perciun¹, Alexandru-Madalin Buse²

¹ Podiatry Clinic, Bucharest, Romania

² Diabetes Department, National Institute of Diabetes, Nutrition and Metabolic Diseases “N.C. Paulescu”, Bucharest, Romania

*Correspondence to: Alexandru-Madalin Buse, National Institute of Diabetes, Nutrition and Metabolic Diseases “N.C. Paulescu”.
E-mail: madalin.buse@yahoo.com. Phone: 0729825499

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Abstract

The non-contact (handheld) infrared thermometers are valuable instruments for evaluating diabetic foot pathology and foot ulcer prevention. Since there is no guideline to indicate the exact way they should be used, clinical judgment helps integrate the collected data in the case management process. Foot care providers must consider that some factors might interfere with the measurement correctness. It has been suggested that in some cases, other investigations besides thermometry have to be performed before making any decisions.

Keywords: Infrared thermometry, diabetes mellitus, Charcot neuroarthropathy, foot care, debatable technique.

Introduction

Diabetic foot problems are mainly the consequence of diabetic peripheral neuropathy, macro- and microvascular disease of the lower limbs, and any other musculoskeletal damage, including infections. Diabetic neuropathic osteoarthropathy is the consequence of damage to sensory, motor, and autonomic nerve fibers. This usually affects people with a long-standing unfavorable metabolic profile.

A redoubtable complication of the diabetic foot is the neuropathic ulceration.

It is thought that 15–20% of diabetics are likely to develop foot ulcerations throughout their long-running disease, becoming thus a public health issue. Once clinically manifested, it is believed to account for 85% of the lower limb amputations [1]. Charcot arthropathy is believed to have a prevalence of up to 1–7.5% in the diabetic population, being probably even higher (29–35%) in neuropathic diabetics [2–4]. Therefore, congruent efforts are

continuously made for both prediction and accurate prevention of diabetic foot ulcerations.

Historically, Hippocrates was one of the first to mention about skin temperature changes, in 400 BC. He claimed that “in whatever part of the body excess of heat or cold is felt, the disease is here to be discovered” [5]. In the modern days (1971/1972), Goller and Sandrow have correlated dermal temperature with some specific areas which were subjected to inflammation and localized pressure and, ultimately, neuropathic osseous fractures [6].

In humans, the feet are not commonly displaying a difference higher than 1°C between corresponding anatomical areas, but the skin temperature on the trunk regularly fluctuates between 33.5–36.9°C, some anatomical regions exhibiting small physiological variations [7].

Many factors may influence skin temperature either diffusely (ambient, hypothalamus, damage of the internal organs) or localized (various conditions affecting either the skin itself or the underlying soft tissues).



Physiologically, there are certain anatomical areas known to display temperature inconsistencies such as body extremities, protruding body parts, close vicinity of the underlying large vessels, or whenever extreme ambient temperature impacts the skin surface. However, cutaneous vasculature does act efficiently [8, 9]. To note, the higher skin temperature related to the diabetic foot injuries has generated research activities which are increasingly numerous to the modern-day.

Various techniques can detect the skin surface temperature, either focusing on some specific anatomical areas as non-contact infrared thermometers do or providing values of the entire sole by using, among others, liquid crystal thermography, a temperature-sensing weighing scale or infrared (IR) camera systems [7, 9].

Although the thermal imaging systems have some important advantages, this paper's main subject will be the non-contact IR thermometer. It is cheaper, user-friendly, largely used by podiatrists, and also having a very good inter-instrument agreement in humans, despite being more time-consuming [1, 5, 6, 10].

The non-contact IR thermometers and scanning technique

Exergen DermaTemp 1001 and Temp Touch dermal thermometers have been considered for a long time, the only investigational and scientifically accepted thermometers for the foot skin measurements. Some observational studies, using four industrial thermometers, showed that these less-expensive devices are reliable tools for home monitoring. To be noted, they also avoid contamination by keeping a safer "distance-to-spot" [1, 5, 11]. Other thermometers may show similar results, but there is a lack of evidence regarding this aspect. However, in the podiatry departments, choosing an IR thermometer might be a challenge, and each practitioner has to deal with many aspects. The IR thermometers have a different range of temperature recording and functioning capabilities. Many of them are not explicitly designed for foot assessment, so we should remember that a damaged foot can

display, compared with the presumed normal contralateral one, a more significant temperature domain. By looking at the ideal IR thermometer qualifications, we could name reliability and validity as an assessment tool, maneuverability, time-efficiency, and the comprehensiveness of its technical book. Without a standardized tool, other devices appeared as substitutes in our daily practice. Therefore, some practical obstacles could arise. Some differences between devices are the calibration frequency, well-timed pauses between measurements, or the number of measurements required at the same site. Besides these, patients have to accommodate at the workroom temperature (24–25°C) by taking off their shoes at least 15 minutes before. However, the appropriate time frame could depend on the potentially asymmetric type of footwear/devices. Whenever certain dressings have previously covered the skin/wound's surface, they should be removed 10 minutes before to regain thermal rebalance [1].

Skin temperature measurements in Charcot's foot

Some researchers found that intense neuropathic foot exhibits higher skin temperature in its entirety but mainly on the dorsal aspect, probably as a consequence of increased blood flow via arteriovenous shunting [12, 13].

From the anatomical perspective, comparing with the sole, on the dorsal aspect, the skin and the subcutaneous tissue are thinner, and the muscle layers are not that complex. It should be mentioned that the thickness of the subcutis fatty layer (which is a real thermal barrier), modulates the transfer of the muscle (or deeper structures) heat to the skin. The lower the subcutis thickness, the higher the skin surface temperature [14]. These facts could also partly explain the findings of the above authors.

Charcot foot may involve one to a few joints/soft tissue areas, making up localized patterns or fully conjoining these in a diffuse picture [9, 15]. The dermal temperature fluctuates according to the stage; it is higher in the active (acute) inflammatory phase, gradually decreases in the subacute phase, and equalizes the unaffected symmetric areas at the end of the healing process

after immobilization. As we might expect, this skin temperature dynamic mirrors the underlying damaged joints.

IR temperature measurements usually involve the plantar projections of the first, third, and fifth metatarsal heads, first metatarsal-cuneiform, talonavicular joints, cuboid, and heel. In fact, these joints are outlining the most vulnerable areas [1, 16]. However, any other region of interest (soft tissue/joint), could be assessed thermally.

As mentioned above, a non-ulcerated Charcot foot displaying a temperature difference $>2^{\circ}\text{C}$ vs. healthy contralateral foot, should be considered in its active stage. This cut-off point has generally been accepted as one of the offloading criteria [9, 17]. However, the practitioners should be aware that values $<2^{\circ}\text{C}$, but higher than normal, could reflect either subclinical persistence of inflammatory activity (argued mainly on imaging), or its full relapse.

The practitioners have to cast the skin temperature criterion aside whenever the Charcot damage is bilateral and synchronously active. Also, whenever subclinical inflammation and soft tissue/osseous infections coexist, an accurate cutaneous temperature evaluation becomes more challenging [1, 9].

It is worth mentioning that a specific stress response (physical activity) of the plantar temperature might predict the conversion from a non-Charcot to Charcot's foot or that Charcot's foot is at-risk for ulcerations [18].

Dermal IR thermometry over at-risk areas, infectious wound, and during the self-monitoring process

Once some (localized) at-risk areas are suspected, it seems logical to focus on them. A diffusely high temperature could synchronously coexist with certain “hot spots”, thus displaying $>2.2^{\circ}\text{C}$ when compared with the contralateral unaffected areas and also suggesting a deeper inflammatory event, probably anticipating clinical lesions.

Once diagnosed, physical activity and plantar pressure must be promptly reduced.

Considerable research efforts were made in IR thermometry as an efficient warning methodology when applied regularly for at-risk areas [7, 16, 19-21].

Some particular entities, which are often encountered as a result of repeated exposure to minor trauma, are the foot's callosities. Becoming hemorrhagic entities, they are converted into pre-ulcerative “hot spots”. The higher abnormal intrinsic vascularity there is, the higher their pre-ulcerative propensity.

A delayed wound healing could be the result of bacterial colonization either in the deeper layers or peri-wound. Among the clinical criteria (STONES: Size, Temperature, Osseous, New area of breakdown, Erythema/Edema, Exudate, Smell), a higher temperature gradient seems to reflect this fact up to 5 times. However, it is not a *sine qua non*-criterion concerning infectious events.

Because a neuropathic foot can display a higher skin temperature on its own and, as a result of repeatedly traumatic events, or even walking, another two STONES criteria must be added to prove infection [1, 18]. This specific situation has the benefit of therapeutic footwear, frequently, for months. Once the ulcer healing steadiness is achieved gradually, the temperature gradient (the affected vs. healthy contralateral matched area) is being replaced by thermal symmetry [11, 16, 21].

There are different examination protocols that frequently select some predefined plantar sites. Although these protocols are very didactic, they do not serve as an absolute rule because almost 52% of foot ulcers are located on the dorsal, lateral, medial, or interdigital surfaces. Moreover, a pre-existing localized plantar forefoot ulceration, which is under repeated pressure, could spread and fistulize to the dorsum of the foot, thus equalizing the temperature of hot spots, and the clinicians should always consider such a provocative sequential event [17, 22]. Thus, we could debate about the apparent rigidity vs. the versatility of the chosen measurement sites.

Home-monitoring for at-risk patients implies that they are specifically educated as well as in full physical abilities. They are greatly encouraged to embrace, regularly,

both self-inspection and dermal temperature scanning. Persistently focal measurement of the vulnerable anatomical sites (foot ulcer recurrence/propensity for new injuries) is an extremely useful self-care maneuver for predicting and further self-prevention practicing [1, 11, 19, 21, 23].

In order to avoid either false positive or negative results, whenever $>2.2^{\circ}\text{C}$ reflects the skin temperature difference between the corresponding locations, individualized thresholds (the baseline temperature difference between the left and the right foot) should be calculated [24].

Several pathologies and other factors that may induce distorted results

- Because of their declivity, the lower anatomical segments- ankle and foot- are the election place for the development of edema in several pathologies, which may also influence the skin temperature.
- The massive non-inflammatory lower limb edema (venous, cardio-renal, and others) could partially disguise more in-depth inflammatory activity, giving falsely lower temperature values.
- The hemiparesis (forearm, leg, and foot) of the contralateral limb after cerebral infarction evolves with lower cutaneous temperature related to its autonomic disfunction.
- Certain asymmetries of the vascular tree could be responsible for asymmetric skin temperature due to different vaso-reflex potency.
- It is known that blood flow influences the skin temperature; however, strict linearity is missing. Whenever the lower limb displays simultaneously venous and arterial disease, at rest, the cutaneous temperature gradient could be higher than normal because of the sluggish venous blood even when the foot pump normally acts during ambulation.
- In venous insufficiency, the higher skin temperature is strongly connected with

lower limbs' inflammation, stasis, and edema [25, 26].

- Effective collaterals, revascularization, vasoactive medication, severe neuropathy, including autonomic disturbances, are factors that may offer unexpected results comparing with a pure severe arterial disease [27, 28].
- Speculating on the asymmetrical blood flow, the dermal temperatures, which are collected above and below the presumed atheromatous obstacle, could suggest an ischemic limb [1]. However, we should remember that the lower limb arterial disease, mainly in diabetes, is rarely fully asymmetric. Thus, the presumed healthy contralateral comparator is missing. Neither IR thermometry nor thermal imaging techniques can offer certainties about lower limb arterial disease complexity. Angiography and ultrasound images are needed.
- The dermal blood flow rate through microvasculature promotes skin temperature values accordingly. Some other factors, which we previously named, although not explicitly related to the device itself, are carrying the risk of distorting the final result [8].

Conclusions

The temperature measurement of the foot with a non-contact IR thermometer is considered an advantageous method for evaluating the diabetic foot, mainly its iconic (complicated or not) Charcot neuropathic osteoarthropathy. This probably unexpected context invites the dedicated caregivers never to exclude any potential influencing and limiting factors, and to ask for additional investigations for better decision making whenever needed.

Conflict of Interest

The authors declare no conflict of interest.

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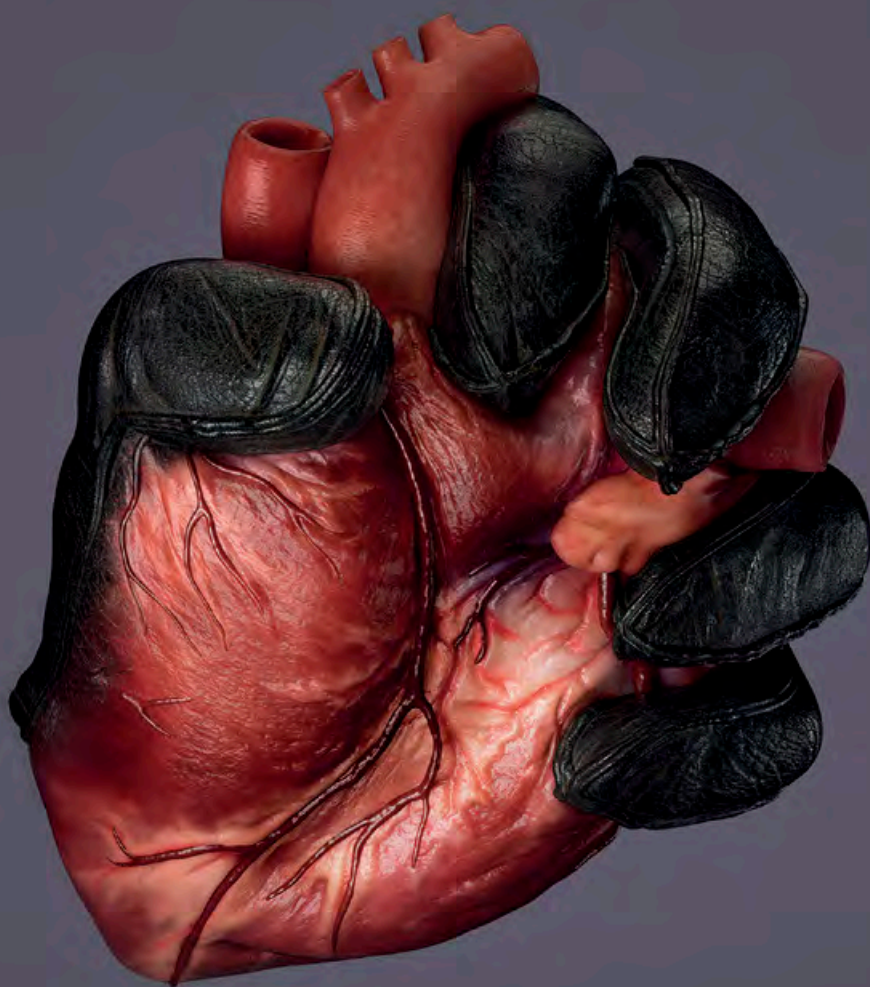
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AJUTAȚI LA REDUCEREA RISCOLUI DE AFECȚIUNI CARDIORENALE LA PACIENȚII CU DZ2





GLUCAGONUL NAZAL - INOVAȚIE ÎN TRATAMENTUL HIPOGLICEMIEI SEVERE

Prof. Dr. Gabriela Roman

Universitatea de Medicină și Farmacie „Iuliu Hațieganu”, Cluj-Napoca
Federația Română de Diabet, Nutriție și Boli Metabolice

Cu toate că insulinele moderne și noile tehnologii pentru monitorizarea glicemiei și administrarea insulinei sunt din ce în ce mai utilizate în practica diabetologică, hipoglicemia rămâne un factor limitant major în atingerea obiectivelor terapeutice la persoanele insulinotratate [1], și în mod special hipoglicemia severă, cu prognostic nefast imediat, dar și pe termen lung [2]. Conform Asociației Americane de Diabet (ADA), hipoglicemia severă sau de nivel 3, este caracterizată prin afectare cognitivă și/sau incapacitate fizică a persoanei afectate, care necesită asistență din partea unei alte persoane pentru recuperare, fără a se menționa o valoare prag a glicemiei [3]. Fiind o complicație acută, cu potențial letal [2], hipoglicemia severă necesită intervenție imediată, iar administrarea de glucagon poate salva viața persoanei afectate. ADA recomandă prescrierea de glucagon tuturor persoanelor cu risc crescut de a dezvolta un episod hipoglicemic semnificativ clinic (definit ca o valoare a glicemiei <54 mg/dl), pentru ca aparținătorii, prietenii sau colegii persoanei afectate să îl poată folosi în caz de urgență [3]. Singurul glucagon disponibil până de curând a fost cel injectabil, ce necesita mai mulți pași pentru preparare, reconstituire și administrare injectabilă corectă [4], în acest caz fiind esențială instruirea riguroasă a persoanei care îl administrează. În situația extrem de stresantă a unei urgențe hipoglicemice, parcurgerea corectă a tuturor pașilor de administrare a glucagonului injectabil de către aparținători poate fi problematică. De asemenea, riscul apariției unei hipoglicemii severe în orice moment sau loc, limitează numărul de persoane instruite să administreze glucagonul injectabil.

Acum, avem la dispoziție glucagonul cu administrare nazală, un polipeptid cu 29 aminoacizi și structură identică cu cea a glucagonului uman și a celui injectabil. A fost dezvoltat pentru a ușura administrarea tratamentului de urgență în hipoglicemia severă, de către aparținătorii persoanei afectate [5, 6]. Glucagonul pulbere nazală 3mg, conține un excipient lipofil, dodecilsulfocolină, care îi amplifică absorbția pasivă prin mucoasa nazală, ceea ce facilitează administrarea sa la o persoană în stare de inconștiență, fără a fi necesară inhalarea [5-8]; este gata de utilizare, nu necesită reconstituire sau preparare, este condiționat în flacon cu doză unică ce poate fi păstrat în

ambalajul original la o temperatură nu mai mare de 30°C, este compact, portabil [5, 6, 9-12] și poate fi utilizat chiar și în prezența congestiei nazale sau a administrării de decongestionante nazale [13].

În studiile clinice randomizate care au comparat glucagonul nazal 3mg cu glucagonul injectabil, la adulți, adolescenți și copii, începând cu vârsta de 4 ani, s-a demonstrat noninferioritatea glucagonului nazal față de cel injectabil în rezolvarea hipoglicemiei induse de insulină [6, 9, 14]. De asemenea, în studii de simulare a administrării la manechin a celor două forme de glucagon, glucagonul nazal a fost administrat corect, cu succes, în majoritatea cazurilor (>90%), atât de către aparținătorii neinstruiți în utilizarea sa, cât și de către cei instruiți, spre deosebire de glucagonul injectabil care a fost administrat corect într-o proporție extrem de redusă (<15%), doar de către cei instruiți pentru a-l utiliza. Timpul necesar administrării cu succes a glucagonului nazal a fost sub 30 de secunde, spre deosebire de câteva minute în cazul glucagonului injectabil [12, 15]. Rezultatele din studiile randomizate și de simulare au fost confirmate și în viața reală la adulți, adolescenți și copii cu diabet zaharat tip 1, în care administrarea glucagonului nazal de către aparținători a dus la rezolvarea majorității episoadelor de hipoglicemie moderată sau severă în decurs de 30 de minute, timpul necesar administrării glucagonului nazal fiind sub 30 de secunde în majoritatea cazurilor [10, 11]. Cele mai frecvente reacții adverse ale tratamentului cu glucagon nazal au fost: gastrointestinale, greață și vărsături (tipice tratamentului cu glucagon), cefalee, iritația tractului respirator superior și creșterea secreției nazale [5]. Pacienții cu risc de hipoglicemie severă trebuie să aibă glucagon disponibil și să fie instruiți împreună cu aparținătorii cu privire la momentul și modul în care trebuie administrat. O analiză a 10 studii a arătat faptul glucagonul nazal are o eficiență similară cu cea a formei injectabile, dar că este preferat de utilizatori datorită ușurinței de administrare [16]. **Aprobarea și disponibilitatea glucagonului nazal oferă o opțiune terapeutică eficientă, cu profil de siguranță favorabil, ușor de administrat, ce poate îmbunătăți tratamentul hipoglicemiei severe.**

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BAQSIMI: singurul glucagon cu administrare nazală

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Dieta și ficatul gras non-alcoolic – o relație de viitor?

Boala ficatului gras non-alcoolic reprezintă o acumulare excesivă de grăsimi la nivel hepatocitar și este principala cauză de boală cronică de ficat la nivel mondial. Ea prezintă o prevalență în creștere, în paralel cu obezitatea, diabetul zaharat de tip II și unele componențe ale sindromului metabolic, mai ales rezistența la insulină.

S-au evidențiat la nivel celular 2 etape patogenice în apariția ficatului gras non-alcoolic; prima este reprezentată de acumularea excesivă de grăsimi la nivelul hepatocitului, și rezistența la insulină, iar cea de-a doua este reprezentată de stresul oxidativ și secreția crescută de citokine proinflamatorii. Prima etapă este impactată de genetică și de balanța energetică pozitivă, iar cea de-a doua de către regimul alimentar.

Termenul de ficat gras non-alcoolic include o serie de componente distincte care variază de la steatoza simplă până la steatohepatita non-alcoolică, urmată în plan histopatologic evolutiv de către fibroză, ciroză și carcinom hepatocelular. La acestea se asociază un risc crescut de boală cardio-vasculară și boală cronică de rinichi.

Dieta alimentară, alături de stilul de viață, joacă un rol cheie atât în dezvoltarea NAFLD, cât și în menținerea și progresia ei. Se insistă tot mai mult asupra impactului pe care îl are dieta din sarcină și perioada de alăptare asupra apariției NAFLD la produsul de concepție.

O schemă nutrițională echilibrată, limitativă și individualizată, va cataliza scăderea ponderală și îmbunătățirea expresiei clinice a bolii hepatice.

Recomandări dietetice specifice NAFLD

1. Scăderea ponderală este recomandată tuturor pacienților supraponderali (cu IMC 25-29,9) sau încadrați într-un grad de obezitate (IMC peste 30 kg/mp). Ea nu trebuie să depășească 1 kg pe săptămână, scăderea rapidă și necontrolată în greutate fiind în detrimentul pacientului și putând chiar să agraveze expresia clinică a NAFLD. Dietele cu conținut caloric foarte scăzut (sub 388 kcal/zi) trebuie evitate, ele putând fi cauza statusului proinflamator sistemic și a creșterii nivelului seric al bilirubinei.

2. Acizii grași saturați induc stres oxidativ și distrucție hepatocitară, cu progresia de la stadiul de steatoză simplă la steatohepatită și apoptoză celulară. Este recomandat un nivel de sub 10% acizi grași saturați în dieta zilnică, care va avea efecte de diminuare a valorilor LDL colesterol și trigliceride; nu se recomandă însă scăderea aportului sub 7%.

3. Acizii grași trans din lactate sunt recomandați pentru consum, în timp ce fracțiunile obținute prin hidrogenarea uleiurilor vegetale induc acțiune proinflamatorie și disfuncție endotelială și sunt de evitat.

4. Acizii grași mononesaturați extrași din ulei de măsline, floarea soarelui, avocado, rapiță, nuci, sunt recomandabili pentru capacitatea lor de creștere a nivelului HDL colesterolului și diminuare a stresului oxidativ la nivel hepatocitar.

5. Acizii grași polinesaturați influențează pozitiv evoluția NAFLD. O dietă cu 2g/zi acizi grași polinesaturați timp de 6 luni a demonstrat scăderea nivelului de steatoză hepatică împreună cu scăderea rezistenței la insulină. Deficitul de acizi grași polinesaturați din dietă induce apariția NAFLD. Dacă fracțiunea omega 3 se regăsește mai ales în uleiul de pește, vegetale cu frunze verzi și semințele de in, acizii grași omega 6 pot fi preluați mai ales din cereale, ouă și grăsimi animale. Raportul recomandat Omega 3-Omega 6 este de 1:1 până la 1:4.

6. Carbohidrații simpli prin consum crescut de băuturi carbogazoase conduc la un risc mai mare de obezitate și apariție a DZ. Alimentele intens procesate au un conținut crescut de zaharoză și fructoză. Fructoza se regăsește mai ales în suc, jeleuri, gemuri și siropuri. Pe lângă conținutul de fructoză, băuturile carbogazoase conțin cantități mari de aspartam și caramel, ambele cu efect proinflamator la nivel hepatocitar. Aspartamul, glucid de 160 de ori mai dulce decât zaharoza, produce creștere ponderală și contribuie la apariția diabetului zaharat. Fructoza induce insulinorezistență și contribuie major la apariția ficatului gras non-alcoolic. Se recomandă din aceste motive un consum maxim zilnic de 360 ml de astfel de băuturi, desi ideală ar fi excluderea completă a acestui tip de consum din dietă.

7. Oligofructoza este un oligomer de fructoză, conținută îndeosebi în cicoare, usturoi, praz, anghinare, cu efecte benefice multiple asupra metabolismului; consumul frecvent ajută la normalizarea valorilor glicemiei și ale trigliceridelor serice. Suplimentarea oligofructozei la supraponderali și obezi induce scăderea valorilor glicemice postprandiale și îmbunătățirea controlului glicemic. Crescând totodată senzația de sațietate. La nivel hepatic oligofructoza atenuează inflamația hepatocitară.

8. Conținutul proteic din dietă are efecte benefice asupra evoluției ficatului gras non-alcoolic. Deficitul proteic și malnutriția pot determina evoluția către steatohepatită. Studiile efectuate pe rozătoare arată că o dietă hiperproteică și hipoglucidică ameliorează metabolismul carbohidraților și reduce steatoza

hepatică datorită inhibării lipogenezei de novo. Aportul de proteine este esențial pentru regenerarea hepatocitară și pune la dispoziție aminoacizi esențiali care previn acumularea în exces a grăsimii intrahepatocitară. O dietă hiperproteică (40% proteine) reduce riscul de boală cardio-vasculară, însă poate induce scleroză glomerulară și hipertensiune renovasculară la pacienții renali cronici. Concluziile studiilor sunt că un aport mediu proteic este cel optim pentru pacienții cu ficat gras non-alcoolic datorită impactului pozitiv asupra scăderii în greutate. 25% aport energetic din proteine nu cauzează efecte adverse.

9. Glutamatul monosodic este un potențator de aromă întâlnit în alimentele procesate, congelate sau conservate și în special utilizat de către bucătăria asiatică. Consumul de glutamat monosodic crește riscul de ficat gras non-alcoolic și potențează inflamația și displazia hepatocitară, crește nivelul de acizi grași liberi și trigliceridemia. Expunerea la glutamat monosodic cauzează deteriorarea oxidării acizilor grași, a sintezei acizilor biliari și interferează cu stocarea lipidelor.

Recomandări nutriționale la pacientul cu ficat gras non-alcoolic sau steatohepatită non-alcoolică

Conținut caloric: 1000-1200 kcal/zi la paciențele obeze

1200-1600 kcal/zi la pacientul obez

Nutrienți în dietă: Carbohidrați 40-50%

Lipide sub 30% din totalul energetic

Proteine aproximativ 20% din total

Deficitul energetic trebuie să fie cuprins între 500-1000 kcal/zi

Acest deficit va permite o scădere ponderală de 0,5-1 kg/săptămână

Necesarul energetic trebuie individualizat. Trebuie să se țină cont de activitatea fizică, înălțime și greutate.

Alegerea produselor alimentare în cazul pacientului cu NAFLD/NASH

- Alimentele pacientului cu ficat gras non-alcoolic trebuie să aibă un conținut scăzut în acizi grași saturați.

- Băuturile răcoritoare cu conținut crescut în sirop de porumb trebuie eliminate complet din dietă.

- Se va consuma doar carne slabă în grăsimi, cu precădere carne albă și doar ocazional carne roșie.

- Alimentele vor fi procesate prin fierbere, la abur sau coapte fără adaos lipidic.

- Se evită prăjirea. Alimentele prăjite sunt mai greu digerabile și au conținut caloric mai ridicat.

- Se interzice consumul de smântână sau răniș.

- Produsele de tip fast-food sunt interzise

- Se recomandă consumul zilnic de 30 g nuci, bogate în acid gras alfa-linolenic, care au capacitatea de a reduce nivelul colesterolului total și a LDL-colesterol.

- Se recomandă consumul de fructe și legume, sursă importantă de vitamine antioxidante (vitamina C, E și beta-caroten):

vitamina C: coacăze negre 184 mg/100 g produs, frunze de pătrunjel 177 mg/100 g, piper roșu 144 mg/100 g, hrean 114 mg/100 g

beta-caroten: morcovi 9938 micrograme/100g produs, piersici uscate 7842 microgr%, pătrunjel frunze 5410 microgr%, frunze de ceapă 3400 microgr%

Vitamina E: ulei de floarea soarelui, germeni de grâu

- Fructele cele mai recomandate sunt sucul de aronia și sucurile proaspăt presate, cu efect potent antioxidant, conținând polifenoli și o cantitate crescută de proanthocianine și anthocianine. Extractul de aronia combate hipertrigliceridemia indusă de fructoză și ajută la scăderea în greutate.

NAFLD este una din cauzele principale de boală hepatică la nivel mondial. Incidența crescută a steatozei simple, cu progresie către steatohepatită și în final ciroză hepatică, trebuie să alarmeze profesioniștii din domeniul sănătății și să îi determine să caute în mod continuu noi oportunități pentru un bun management al afecțiunii. La momentul prezent știm că adevărata cale către controlul bolii ficatului gras non-alcoolic este mai degrabă modificarea stilului de viață, cu accent pe dieta alimentară, decât terapia medicamentoasă. Depinde de noi, ca specialiști în domeniul sănătății, să educăm pacientul cum să își gestioneze eficient afectarea hepatică acasă, la locul de muncă și în fiecare moment din viața de zi cu zi.

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